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*It is characteristic of Science and Progress that they
continually open new fields to our vision—*PASTEUR

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THE ANTIGENICITY OF CRYSTALLINE LYSOZYME

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The first attempt to produce lysozyme antiserum seems to have been that of Jermoljewa and Bujanowskaja (1931)   Unpurified lysozyme was used   Roberts (1937) described results obtained with a purified but not crystalline lysozyme preparation   He reported that lysozyme is antigenic, that precipitins are formed, and that rabbit antiserum inhibits lysozyme activity on *Micrococcus lysodeikticus*   Fleming and Allison (1922) reported that serum had the property of lysing *M lysodeikticus*   Roberts did not mention whether this characteristic of serum interfered with his inhibition tests   The following communication gives the results of experiments in which recrystallized (4 to 6 times) egg white lysozyme was utilized   The lysing property of normal serum was taken into consideration

MATERIALS AND METHODS

Antigen   Two lysozyme preparations from egg white were used in this study   Both were crystalline   One was prepared by the method of Alderton *et al* (1945) and was recrystallized six times   The other was prepared by the method of Alderton and Fevold (1946) and was recrystallized four times

Sera   Antisera were prepared in chinchilla rabbits either by repeated intravenous injection, or by subcutaneous injection of a water in oil emulsion according to the method of Freund and McDermott (1942)

Test for lysozyme activity   A suspension of *M lysodeikticus* was distributed into a number of vials and dried from the frozen state   For each test a fresh vial was opened and planted on two nutrient agar slants   These were incubated at 37 C for 16 hours and the growth was washed off with 0 85 per cent NaCl and centrifuged   The sediment was suspended in 0 85 per cent NaCl to give a turbidity reading of 100 on the Klett-Summerson photoelectric colorimeter using a no 42 filter   One ml of this living suspension was added to 1-ml volume of test solution, and the tubes were placed at 37 C   Readings were taken at varying time intervals, for the purpose of this study, readings at $4\frac{1}{2}$ hours were selected as most suitable, as they gave optimal lysis and eliminated potential complication due to contaminants

Inhibition of lysozyme with specific antiserum   To the varying concentrations of crystalline lysozyme in 0 5-ml volume were added 0 5 ml of varying dilutions of serum   The tubes were shaken, placed at 37 C for 1 hour, and then to each mixture 1 ml of *M lysodeikticus* suspension (described under test for lysozyme activity) was added   The tubes were shaken and placed at 37 C, and the extent of lysis was recorded at $\frac{1}{2}$, 1, 2, 3, 4, and 5 hours

Precipitin test   These tests were made by mixing 0 4 ml of antigen in the proper dilution with 0 4 ml of undiluted antiserum   The tubes were placed at 37 C for 1½ hours, transferred to the refrigerator overnight, and the amount of precipitate was estimated after centrifugation

EXPERIMENTAL RESULTS

Lysozyme activity of normal rabbit serum   In corroboration of Fleming's original observation (1932) it was found that rabbit sera exhibited varying degrees of

TABLE 1
Normal rabbit serum lysozyme activity
(4 5-hour readings)

RABBIT	FINAL SERUM DILUTION		
	1 67	1 67	1 670
S-1	+	±	0
S-2	0	0	0
S-3	+	±	0
S-4	+	±	0
S 5	±	0	0
S 6	±	0	0
S-7	±	±	0
S-8	+	±	0
S 9	+	±	0
S-10	+	±	0
S 33	±	±	0
S-0	+	±	0
S-1011	+	±	0
S 170	+	±	0
S-177	+	±	+
S 182	+	±	0
S 1477	+	±	0
S-1479	+	±	0
S 1974	+	±	0
S-196	±	±	0

0 = no lysis, ± = partial lysis, + = complete lysis

lytic activity against *M lysodeilticus*   One hundred and three normal rabbits were bled from the marginal ear vein, the serum was separated and tested for lytic activity   Three dilutions of serum each in a volume of 0 3 ml were employed (0 3, 0 03, and 0 003 ml), the diluent being extract broth   To the contents of each tube, 0 7 ml of broth and 1 ml of *M lysodeikticus* suspension (described under Materials and Methods) were added, and the tubes were shaken, placed in the 37 C incubator for 4½ hours, and read   The degree of resulting lysis was recorded   The results of a representative number of such tests are shown in table 1

Rabbit serum S-2 seemed to have no lytic activity   Three rabbits out of a

total of 103 were found to be in this category   It is interesting that this lytic property of serum seems to be inherent in the eight species of animals tested including humans   The role of the active constituent and the reason for its absence in a small percentage of rabbit sera are obscure

Preparation of antilysozyme sera in rabbits   A series of 12 intravenous injections (total of 17 mg of crystalline lysozyme each) were made in two rabbits   No

TABLE 2

Rabbit antibody response to combined intravenous lysozyme immunization and saline in oil emulsion

SERA	FINAL ANTIGEN DILUTION					
	1 10^2	1 4 × 10^2	1 16 × 10^2	1 64 × 10^2	1 256 × 10^2	1 1024 × 10^2
51	0	0	0	0	0	0
52	0	±	1	4	4	1
NRS	0	0	0	0	0	0

4 = large amount of precipitate with perfectly clear supernatant, 3, 2, 1 = decreasing amounts of precipitate with decreasing clarity of supernatant, ± = trace, 0 = no precipitate, NRS = normal rabbit serum (control)

TABLE 3

Comparison of precipitin response with the lytic activity of serum

SERA	PRECIPITIN TITER			LYSOZYME ACTIVITY			
	Final Lysozyme Dilution			Final Serum Dilution			
	1 50 × 10^2	1 200 × 10^2	1 800 × 10^2	1 6 6	1 66	1 666	1 6 666
$\frac{1}{2}$	tr	tr	1	±	0	0	0
12	—	3	2	±	0	0	0
S-2	4	0	0	0	0	0	0
101	—	0	0	+	0	0	0
112	4	4	3	+	±	0	0
104	0	1	1	+	±	0	0
108	0	0	0	+	±	0	0
109	4	1	0	+	0	0	0
111	0	2	1	±	0	0	0
NRS	0	0	0	±	0	0	0

0 = no lysis, ± = partial lysis, + = complete lysis, tr = trace

precipitating antibodies were observed in sera obtained at varying time intervals   A water in oil emulsion containing 15 mg of lysozyme was then prepared and injected subcutaneously into one of the two rabbits (no 52)   The other rabbit (no 51) received 6 more intravenous injections totaling 18 mg of lysozyme   One week later both rabbits were bled (interval of 3 weeks for no 51 rabbit) and precipitin tests made   The results are shown in table 2

Another series of four rabbits received 15 intravenous injections totaling 35 mg of lysozyme each   Only 1 of the 4 sera gave a positive precipitin titer, this

normal rabbit sera have the property of lysing *M lysodeikticus* and there seems to be no relationship between inherent serum lysozyme activity and the production of lysozyme antibodies   Antiserum prepared against one lysozyme inhibits the lytic activity of only its specific lysozyme, despite the fact that both lysozymes exhibit apparently similar lytic activities   This again demonstrates species-specific differences   Further, it indicates that the chemical groupings, responsible for lytic activity, may differ in the two lysozymes, since the lytic activity of the egg white lysozyme may be completely inhibited, whereas the serum lytic activity is apparently unchanged   There is, of course, always the possibility of a physical blocking of the active lytic groups when antigen-antibody union is effected

In view of the above it might be desirable either to define individual lysozymes (any material causing the lysis of *M lysodeikticus*) more fully, or to choose a more appropriate designation for each individual lysozyme   The term "lysozyme" itself, as pointed out originally by Fleming, is ambiguous and certainly not descriptive of any one substance

Crystalline lysozyme produces partial lysis of *M lysodeikticus* in dilutions of $1\ 15 \times 10^6$ to $1\ 20 \times 10^6$ Serum gives partial lysis in dilutions of about 1 100 If one assumes that the activity per unit weight of each lysozyme is roughly of the same magnitude, then it would appear that the concentration of lysozyme in serum is of the order of 0 01 per cent   It would thus seem that lysozyme is a minor constituent of serum protein, the significance of which is as yet undetermined

The use of crude egg white lysozyme in man has recently been reported (Ponomareva, 1946a, 1946b)   In view of the findings presented here, it might be well to exercise caution in the human experiments since the possibility of sensitization is obvious

SUMMARY

Crystalline lysozyme prepared from egg white is antigenic   It combines with specific rabbit antisera to high titer   Antilysozyme rabbit serum inhibits the activity of lysozyme on *Micrococcus lysodeikticus*

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yellow oil which showed strong bacteriolytic powers and appeared to be an acid More recently, Hays and co-workers (1945) have separated a number of active lipoidal fractions, which they have called the "Pyo" compounds

PIGMENT PRODUCTION IN VARIOUS MEDIA

In the present studies, cultures of *P aeruginosa* from five different sources were used B, from human feces, S, from a human throat, Ha, from the infected cheek pouch of a hamster, H and T, stock cultures from two different laboratories Except for slightly more rapid pyocyanin production by strain B, the reactions of all five were identical in the experiments to be described, so no further mention of strain will be made

TABLE 1

Pigment production and acidity in various media

MEDIUM	pH	PIGMENTS	
		Pyocyanin	Fluorescent
0 5% Glucose	8 12	+	+
1 0% Glucose	6 20	−	−
1 5% Glucose	4 80	−	−
2 0% Glucose	4 18	−	−
0 5% Glycerol	8 50	+	+
1 0% Glycerol	7 57	++	++
1 5% Glycerol	8 10	++	++
2 0% Glycerol	8 26	++	++
Potato + glycerol	8 35	++++	−
Veal infusion	8 40	+	?
Veal infusion + glycerol	8 36	+	?
Blood	8 28	+	?

Nutrient extract broth was used as the base medium with the supplements listed   Cultures were incubated 2 weeks at 37 C

The first series of experiments was done to determine the effect of various media on pigment production   Nutrient extract broth was used as a base, and the medium was designated by whatever other ingredients were added 0 5, 1 0, 1 5, and 2 0 per cent glucose, 0 5, 1 0, 1 5, and 2 0 per cent glycerol, potato+ 1 0 per cent glycerol, veal infusion, veal infusion + 1 per cent glycerol, and 5 per cent whole blood   The results, including the final pH of each culture, are shown in table 1 Of the five pigments mentioned above, only pyocyanin and the fluorescent pigment are given, because pyorubin was not formed by any of the strains used, and α-oxyphenazine and the brown substance appear to be formed, not directly by the organism but indirectly from pyocyanin and the fluorescent pigment, respectively   Pyocyanin was determined by shaking the culture to oxidize the leucobases of the pigments, and extracting with chloroform   Fluorescence was noted on dilution of a small amount of the culture after chloroform extraction

It appears that pigment production is associated with alkalinity, and if enough

glucose fermentation takes place so that the reaction remains acid no colored substances are formed   Cultures containing small amounts of glucose—up to 1 per cent—show an acid reaction during the first week of incubation, and then gradually become alkaline, with some pigment formation   That the pigments are not already present in some colorless form is shown by the fact that when NaOH is added to an acid culture and the pH brought to 8 0, there is no immediate evidence of pigment, but in a few days both pyocyanin and the fluorescent material begin to appear   The slight amounts of pigments formed in veal infusion and blood broths, which give distinctly basic cultures, indicate that alkalinity is not the only factor involved, and that enriched media of this type inhibit rather than encourage pigment production, even in the presence of glycerol   The addition of glycerol alone favors the production of both pigments, and potato further increases pyocyanin formation, but appears to inhibit the fluorescent material   (The reaction of all these cultures is remarkably constant after the first 2 weeks, 10-week cultures, either acid or basic, show the same pH as at 2 weeks, although such properties as color and viscosity may have changed considerably   In no case do the organisms die out)

ANTIBIOTIC ACTIVITY IN VARIOUS MEDIA

Three of these media were chosen for further work 1 5 per cent glucose, in which no pigments were produced, potato + 1 per cent glycerol, in which pyocyanin was formed, and 1 per cent glycerol, which yielded both pyocyanin and the fluorescent substance   Samples were taken from cultures after 1, 4, and 10 weeks of incubation, each was sterilized by boiling for 1 minute and 3 ml were added to a tube of melted nutrient agar   Plates were poured and streaked with three test organisms, *Staphylococcus aureus*, *Escherichia coli*, and *Mycobacterium smegmatis* After 24 hours the growth of *S aureus* and *E coli* on the test plates was compared with that on a control plate of nutrient agar   Because *M smegmatis* grows more slowly, both test and control plates were replaced in the incubator for another 24 hours before the growth of that organism was recorded   The results are given in table 2   It is clear that the 1 5 per cent glucose culture, producing no pigments, is also lacking in antibiotic activity, whereas the other two media are effective in both respects   The diminishing inhibition of *E coli* by older cultures will be referred to later

PROPERTIES OF CULTURE FRACTIONS

In order to analyze further the relation between pigment formation and antibiotic potency, cultures in the three media were allowed to incubate for 3 weeks and then fractionated   The 100-ml broth culture was shaken in order to oxidize the leucobases of both pyocyanin and the fluorescent pigment   It was then extracted with chloroform (4 successive 20-ml portions) to remove pyocyanin and any small amounts of α-oxyphenazine present   Because pyocyanin breaks down rapidly into the latter substance in chloroform solution, this extract was immediately treated with very dilute hydrochloric acid (three 10-ml portions), pyocya-

antibiotic material from acidified cultures by ether (matter which is not extracted from alkaline cultures by chloroform), thus confirming the fatty acid hypothesis, and the presence of some water-soluble inhibitory substance, not hitherto described, in residues containing the fluorescent pigment

This last observation is especially significant in view of the statements

TABLE 4

Effect of time on the antibiotic activity of culture fractions

WEEK	AMT	WHOLE CULTURE			PYOCYANIN			OXYPHENAZINE			ETHER EXTRACT			FLUORESCENT RESIDUE		
		S aureus	E coli	M smeg matis	S aureus	E coli	M smeg matis	S aureus	E coli	M smeg matis	S aureus	E coli	M smeg matis	S aureus	E coli	M smeg matis
	ml															
1	4	−	−	−	−	−	−	−	+++	−	−	+++	−	−	+++	−
	3	−	−	−	−	−	−	−	+++	−	−	+++	−	−	+++	−
	2	−	++	−	−	−	+	−	+++	−	+	+++	−	+	+++	+
	1	−	++	−	−	−	+	++	+++	−	++	+++	+	+	+++	+
	0 5	+	+++	−	+	+	−	+++	+++	+	+++	+++	++	++	+++	++
2	4	−	−	−	−	−	−	−	+++	−	−	+++	−	−	+++	−
	3	−	−	−	−	−	−	−	+++	−	−	+++	−	−	+++	−
	2	−	−	−	−	−	−	++	+++	+	+	+++	−	−	+++	−
	1	−	+	−	−	+	−	++	+++	+	++	+++	+	−	+++	−
	0 5	+	+++	−	+	+	−	+++	+++	++	++	+++	+	+	+++	−
3	4	−	−	−	−	−	−	−	+++	−	−	+++	−	−	+++	−
	3	−	−	−	−	−	−	−	+++	−	−	+++	−	+	+++	−
	2	−	−	−	−	−	−	−	+++	−	+	+++	+	+	+++	−
	1	−	−	−	−	−	−	++	+++	+	++	+++	+	++	+++	+
	0 5	+	++	−	−	+	−	++	+++	+	++	+++	+	++	+++	+
4	4	−	+	−	−	−	−	−	+++	−	−	+++	−	+	+++	−
	3	−	+	−	−	−	−	−	+++	−	−	+++	−	+	+++	+
	2	−	++	−	−	+	−	+	+++	−	+	+++	−	++	+++	++
	1	−	++	−	−	+	−	++	+++	+	+	+++	−	+++	+++	+++
	0 5	++	+++	+	+	++	+	++	+++	++	++	+++	+	+++	+++	+++
6	4	−	+	−	−	+	−	−	+++	−	−	+++	−	+	+++	+
	3	−	++	−	−	+	−	−	+++	−	−	+++	−	+	+++	+
	2	−	++	−	−	++	−	+	+++	+	−	+++	−	++	+++	+
	1	−	+++	−	+	+++	+	++	+++	++	+	+++	+	++	+++	++
	0 5	+	+++	+	++	+++	++	++	−++	+++	++	+++	+	+++	+++	++
10	4	−	+++	−	−	+	−	−	+++	−	−	+++	−	+	+++	−
	3	−	+++	−	−	++	−	−	+++	−	−	+++	−	+	+++	+
	2	−	+++	−	−	+++	−	+	+++	+	−	+++	−	++	+++	++
	1	−	+++	+	+	+++	+	++	+++	++	−	+++	−	++	+++	++
	0 5	+	+++	+	+++	+++	+	+++	+++	+++	+	+++	+	+++	+++	++

(Hettche, 1932, Schoental, 1941) that all antibiotic material produced by this organism is removed by fat solvents The substance is heat-stable, and it seems likely that it is identical with the inhibitor produced by other fluorescent bacteria Antibiotic activity in this group of organisms has been described by several authors Garré (1887) used *Pseudomonas putida* to inhibit *S aureus* and other organisms, and Frost (1904) found both *Pseudomonas fluorescens* and *Pseudomonas putida* bactericidal for *Eberthella typhosa* Lewis (1929) showed

that *P fluorescens* was effective against sporeforming soil bacteria and micrococci, but not against *E coli* or *Serratia marcescens*

THE EFFECT OF TIME ON PRODUCTION OF PIGMENTS AND ANTIBIOTIC ACTIVITY

To determine the effect of time on the production of these fractions, cultures in glycerol broth were analyzed after 1, 2, 3, 4, and 10 weeks of incubation   The results, shown in table 4, indicate that several changes occur as the time of incubation is increased   The inhibition of *E coli* by whole cultures and by pyocyanin decreases rapidly after the third week   A comparison of the effect on
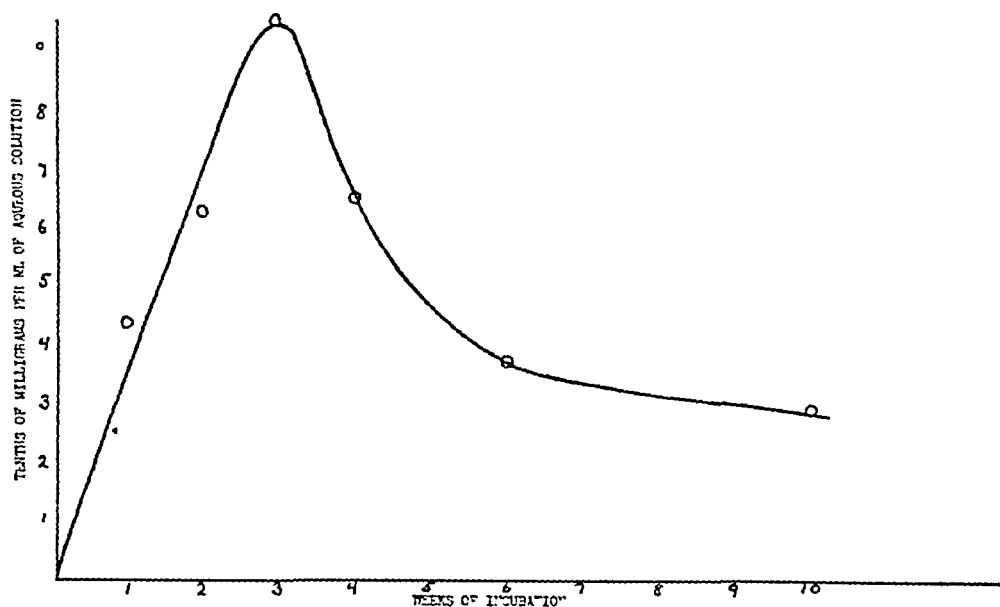
FIG 1 RELATION BETWEEN PYOCYANIN CONTENT AND AGE OF CULTURE
(ONE PER CENT GLYCEROL BROTH MEDIUM)

this organism with the actual concentration of pyocyanin (figure 1) shows a direct proportion   The actual amount of α-oxyphenazine extracted is small and proportional to the amount of pyocyanin   The ether extract, on the other hand, shows a growing effectiveness in older cultures   An increase in this factor at the time when pyocyanin is decreasing would explain the continued inhibition of *S aureus* and *M smegmatis* by whole cultures   The activity of the fluorescent residue is greatest in young cultures, diminishing after the second week.

SUMMARY

Pseudomonas aeruginosa produces no pigments in culture media containing sufficient glucose (over 1 per cent) to establish and maintain an acid reaction Pyocyanin is profusely formed in potato glycerol broth, and in glycerol broth both pyocyanin and a fluorescent pigment are produced   Enrichment of the medium

THE MODE OF ACTION OF NITROFURAN COMPOUNDS

II APPLICATION OF PHYSICOCHEMICAL METHODS TO THE STUDY OF ACTION AGAINST STAPHYLOCOCCUS AUREUS

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The first report of this series called attention to the fact that, of a group of varied nitrofurans, the 2-(5-nitro)-furaldehyde semicarbazone, named "furacin," was distinctly different from the others in its mode of antibacterial activity when tested with a coagulase-positive strain of *Staphylococcus aureus* (Cramer and Dodd, 1945) We have further examined the effect of furacin upon the oxidation-reduction potential of growing cultures of a coagulase-positive staphylococcus, and also the effect of eventual growth upon the concentration of furacin We have found that a poising of E_h does occur, and that subsequently if growth takes place the chemical compound is reduced, undoubtedly at the 5-nitro group We wish to present data related to these events, and to discuss the implications with respect to the vital processes of the bacteria

These data have been obtained by the application of purely physicochemical methods, and at least in the instance of the polarographic method of analysis of bacterial cultures, represent an unusual and simple approach to the determination of a single constituent in the complex mixture that results from bacterial growth, without the necessity of detailed separation procedures

EXPERIMENTAL

The changes of potential occurring in growing cultures were measured by means of a simple potentiometer, a Leeds and Northrup type K_1 (Hewitt, 1936) Electrodes were made by sealing 22-gauge platinum wire into 2-mm soft glass tubing, mercury was used to connect the Pt electrode to the potentiometer circuit The culture vessels were 25-mm pyrex test tubes The electrode and an inverted U-tube, the latter to serve as an electrical bridge, were rolled into a cotton plug and sterilized by autoclaving, following which the bridge was filled aseptically with a sterile, saturated KCl solution containing 3 per cent agar For experimental observations exactly 25 ml of the medium to be examined were placed in a sterile culture vessel and inoculated with 50,000 to 100,000 organisms per ml, and the electrode- and bridge-containing plug was inserted The vessel was then placed in a water bath at 37 C, the outer tip of the KCl-agar bridge being placed in a reservoir of saturated KCl solution Into this reservoir also was placed the side arm of a saturated calomel electrode, and the cell thus formed was connected to the potentiometer It was determined that the calomel half-cell under the

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Change in concentration of furacin during growth   Table 2 and figure 2 show current-voltage data for broth—broth in which *S aureus* has grown fully and broth containing furacin at a concentration of 1 100,000

TABLE 2

Current-voltage relations for furacin in broth

EMF , VOLT	BROTH	BROTH AFTER FULL *S aureus* GROWTH	FURACIN 1/100,000 IN BROTH
0 0	−3 3	−3 1	−3 3
0 2	−1 5	−1 5	−1 6
0 3	−1 4	−1 4	−1 4
0 4	−1 3	−1 35	−1 3
0 5	−1 3	−1 3	−0 7
0 6	−1 3	−1 3	−0 6
0 8	−1 05	−1 05	−0 35
1 0	−0 75	−0 65	0 0
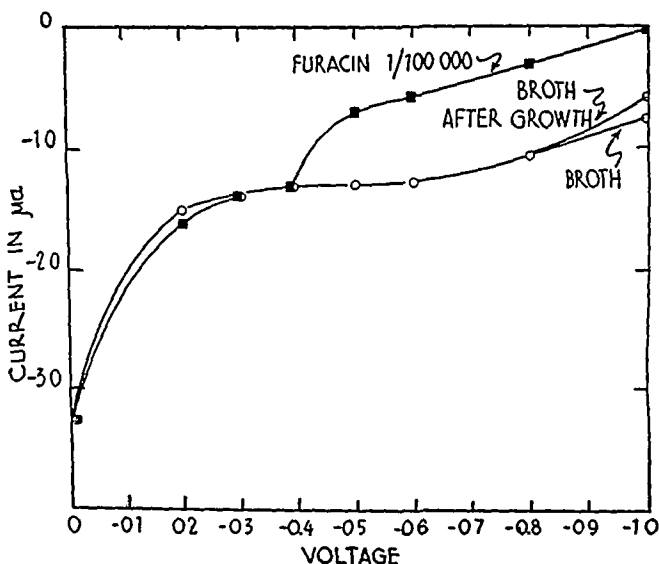
FIG 2 POLAROGRAMS FOR BROTH AND 2-(5-NITRO) FURALDEHYDE SEMICARBAZONE IN BROTH
-O—O- broth
-■—■- 2-(5-nitro) furaldehyde semicarbazone in broth, concentration, 1 100,000

The effect of added furacin is obvious   The average difference in the diffusion current at −0 8 volts due to the presence of 1 100,000 furacin is 0 7 μa, i e , $\triangle$ $i_d^{0\ 8} = 0\ 7\ \mu a = 1\ 100,000$ Similarly, we have determined that for a concentration of 1 50,000 $\triangle$ $i_d^{0\ 8} = 1\ 4\ \mu a$, and for 1 25,000 $\triangle$ $i_d^{0\ 8} = 2\ 75\ \mu a$ Thus, for this range of concentration, the amount of furacin is a linear function of $\triangle$ $i_d^{0\ 8}$ The average value for the dilution of furacin producing $\triangle$ $i_d^{0\ 8} = 1\ \mu a$

under these conditions is 1 70,000   This is the quantitative analytical basis for estimating furacin in bacterial culture media

Table 3 shows representative data for the amount of furacin remaining in a culture of *S aureus* growing in the presence of 1 100,000 furacin, visible growth indication is also included

<div align="center">

TABLE 3

Changes in concentration of furacin during growth

(Initial concentration furacin = 1 100,000)

</div>

HOURS AFTER INOC	Δ id^{2}, μa	RESIDUAL FURACIN	VISIBLE GROWTH
		%	
0	0 7	100	— —
2	0 7	100	— —
4	0 7	100	— —
6	0 65	93	— —
8	0 55	79	— —
10	0 50	71	— —
12	0 45	65	trace
15	0 25	36	++
18	0 15	21	++++
24	0 10	14	++++

DISCUSSION

It has long been known that in broth *S aureus* has a lag period of 1 to 2 hours, followed by a rapid growth that is complete in 8 to 9 hours   The effect of furacin in a concentration of 1 100,000 is to prolong the lag period, in this case the prolongation being at least 6 to 7 hours   We have now demonstrated that during this prolonged lag the E_h of the culture remains poised   Moreover, during subsequent growth, furacin disappears

The poising of potential, followed by what seems to be a normal drop in E_h, confirms our previous conclusion (Cramer and Dodd, 1945) that the only effect observable in population curves of this particular drug-organism combination occurs during the lag phase   It may well be that the poising of the E_h at a point unfavorable to growth is the primary cause for bacteriostasis, in line with the suggestion of Dubos (1929) concerning crystal violet

It is also apparent that an induction period is necessary before the reduction of the 5-nitro group of the furacin molecule occurs (table 3)   With furacin at a concentration of 1 100,000 the organism under these conditions has a lag period of 8 to 9 hours   Reduction, however, is initiated 5 to 7 hours after inoculation, and at 9 hours is approximately 25 per cent complete   This suggests that reduction is initiated shortly before the lag period is ended   This would appear to indicate that at least the initiation of reduction may be necessary before growth can take place, and may signify that furacin must be reduced below a critical level before growth starts

CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

II Changes in Reactions of Staphylococcus aureus to Vital Dyes[1]

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In a previous paper we described the gross appearance of penicillin assay plates after chemical treatments which reveal sharp boundaries around the zones of inhibition (Dufrenoy and Pratt, 1947) Most of the tests that were described are effective following exposure of the test organisms to penicillin for periods as short as 2 to 3 hours, i e , before zones of inhibition are discernible on untreated plates Several of the tests may find application as rapid cylinder plate assay methods (Pratt and Dufrenoy, 1947) The sharp delineation of the inhibition zones was interpreted as an expression of a threshold effect, positive reactions for —SH groups and for OH=C—C=OH groups were obtained outside the zones of inhibition, but not inside The evidence suggested an increase of rH within the zones and that this increase was correlated with inhibition of dehydrogenase systems Since there is strong evidence in the literature that dehydrogenase activity depends on the structural integrity of the microorganism (Guggenheim, 1945), it seemed of interest to study the changes in response to various reagents in different parts of the cells of test organisms following exposure to penicillin In this paper cytochemical structure will be interpreted from microscopical study of living cells of *Staphylococcus aureus* treated with vital stains, and from observations made after treatment of the organisms with various other chemical reagents Our experimental data confirmed the data available in the literature to the effect that the part within the living cells of *Staphylococcus aureus* which responds most evidently to various reagents represents the vacuolar materials

The accumulation of vital dyes in the vacuole may be correlated with the presence and nature of phenolic compounds within the vacuolar solution in a twofold manner first, because phenolic compounds, and in general dienol compounds, play a fundamental role in the respiratory systems providing the energy for active absorption, secondly, because some actual linkage may occur Historically it may be noted that as early as 1900 Nakanishi recognized that living and dead staphylococci respond differently to staining with methylene blue

Imsenecki (1946) has pointed out that "many authors, Piekarski, Peshov, Robmorv,—found in long cells of *Proteus vulgaris*, chromophilic granules which they regard as nuclei the structures mistaken for nuclei are polar granules,

cal process entailing expenditure of energy that is derived from respiration, as respiration becomes unbalanced, the vacuolar solution not only becomes unable to absorb materials from the external environment, but even becomes unable to retain the solutes it already contains   Thus its solutes are free to seep out   This condition, which is one of the early symptoms of the effect of penicillin on susceptible cells, occurs concomitantly with the disorganization of the cellular nucleoproteins and the liberation of lipids and fatty acids

The disorganization of cellular constituents is notably revealed by the "lipophanerese," defined by Lison (1936) as the unmasking of the reactive groups of the fatty components from the liponucleic complex   Hurst (1945) pertinently remarked that "lethality usually involves an irreversible increase in phenoloxidase activity, produced by the displacement of protective lipoids from the tissue receptors "

Data previously reported from assay plates (Dufrenoy and Pratt, 1947) demonstrated that bacteriolytic or bacteriostatic effects of penicillin are correlated with a relative increase of phenoloxidase activity that occurs concomitantly with an inactivation of dehydrogenase systems   This inactivation may be cytochemically visualized as resulting from displacement of the protective lipoids from the lipoproteins in the dehydrogenase systems

In the present investigation it has been shown experimentally by evidence from standard 16-hour assay plates treated with appropriate reagents (Nile blue, copper acetate, or fat-soluble dyes) that lipids are displaced from cells undergoing lysis in the inhibition zones surrounding cylinders containing solutions of penicillin ranging in concentration from 0 25 to 8 units per ml, and that the liberated lipids are hydrolyzed into fatty acids   It is well known that basic dyes which are able to combine with fatty acids form soaps which have the color of the salt of the dye   The sharpest response was obtained with Nile blue, which Knaysi (1941) recommended for the discrimination of neutral fats from fatty acids

When standard 16-hour assay plates are flooded for 1 minute with a saturated aqueous solution of Nile blue, and then are rinsed with distilled water, normal colonies (uninhibited areas of the plates) stain a deep blue and stand out boldly from the agar substrate   Where lysis has occurred, within the zones of inhibition, a purple color develops   This area of lysis is surrounded by a clear blue ring corresponding to the region of enhanced growth just beyond the threshold of bacteriostatic concentration of penicillin

A convergent line of evidence that fatty acids are liberated at the site of bacteriolysis is furnished by flooding 16-hour assay plates with a saturated aqueous solution of copper acetate and further incubating them for 6 hours at 37 C   When this is done, a thick, opaque layer of bluish copper salts of fatty acids develops covering entirely the areas of the inhibition zones   The areas of uninhibited growth do not appreciably react with the reagent   The reactive and nonreactive regions are sharply delineated

Further support for the conclusions drawn from the results obtained with Nile blue and copper acetate was provided by experiments with FD and C yellow

no 3,[3] a fat-soluble dye which stains neutral fat a bright yellow and imparts a deep orange color to fatty acids   Plates flooded with a saturated solution of the dye in methylal develop a bright orange color where lysis has occurred   Each zone of lysis is surrounded by a bright yellow ring delineating the region of enhanced growth

DISCUSSION AND CONCLUSIONS

In previous papers (loc cit) we demonstrated that suitable reagents applied to penicillin assay plates under appropriate conditions sharply delineate the general background of uninhibited growth from the zones of inhibition surrounding cylinders containing penicillin   The results were interpreted as evidence of a threshold for—SH vs S-S groups or for dienols vs diketones in the uninhibited and inhibited areas   It has been known for some time that—SH groups are essential metabolites for *Staphylococcus aureus* and that the blocking of —SH groups inhibits the growth of the staphylococci (Fildes, 1940)   Our experimental data from cylinder plate assays, therefore, suggested that penicillin may act through this mechanism, i e , by blocking —SH groups, thus lending biological support to the chemical evidence presented by Cavallito (1946)

We have also observed (unpublished experiments) that after exposure to bacteriostatic concentrations of penicillin cells of *S aureus* are no longer gram-positive   This result is consistent with the findings of Henry and Stacey (1946) concerning the significance of the —SH group in the gram-positive complex Further evidence that —SH groups may be involved in the interference of penicillin with the growth of *S aureus* can be marshaled from the observation of Gale and Taylor (1946) that penicillin prevents the assimilation of glutamic acid, one of the constituents of glutathione

In the present paper discussion is confined to phenomena that are revealed by staining and that may be considered to be incidental to changes affecting the sulfhydryl compounds   Active absorption of solutes by living cells may be assumed to entail expenditures of energy provided for by aerobic respiration which depends upon the cytochemical integrity of liponucleoproteins involving —SH   Therefore, interference of penicillin with —SH components of the respiratory systems might be postulated to effect (1) changes in the rate of absorption of solutes, and (2) swelling of the organisms coincident with the disorganization of the liponucleoproteins and liberation of lipids and fatty acids

The results of our experiments tend to support these hypotheses   Our evidence obtained from vital staining of cells of *S aureus in situ* on the assay plates or after transfer from different regions of the plates to a drop of the dye solution on a slide shows that, following exposure to bacteriostatic concentrations of penicillin, the cells lose their ability to accumulate neutral red, methyl green, or meythlene blue within the vacuolar solution, and that they swell to at least twice their original diameter   Evidence was also obtained that concomitant

[3] Sold by National Aniline Division, Allied Chemical and Dye Corporation, Buffalo, New York

with or subsequent to the swelling of the cells, fatty acids appear   These may account for the downward shift of pH revealed by the use of indicators (Dufrenoy and Pratt, 1947)

The cytological observations reported in this paper are mainly from plates seeded and preincubated for 3 hours on which penicillin was subsequently allowed to diffuse during a 3-hour secondary incubation period, since in that short time bacteriostatic effects were obtained without extensive bacteriolysis in the inhibition zones   The standard 16-hour plates were not used routinely for the cytological observations, since, because of the extensive bacteriolysis that occurred in that length of time, it was difficult to find material suitable for study The 3-hour technique described above more readily provided cells appropriate for our different studies   The longer diffusion period, on the other hand, was found to provide the best material for the study of lipids and fatty acids arising from bacteriolysis

SUMMARY

In continuation of work previously reported, studies of penicillin assay plates have been made by means of techniques intended to delineate the cytochemical picture that develops on such plates when the test organisms are subjected to the action of penicillin

The present paper concerns cytochemical changes that occur in different parts of bacterial cells exposed to bacteriostatic or bactericidal concentrations of penicillin

The first evidence of the effect of penicillin on *Staphylococcus aureus* was observed to be the failure of the dividing organism to apportion vacuolar material to daughter cells

This was followed by failure of the vacuoles to retain material normally encompassed therein

These changes were manifest in cells under the influence of penicillin, first by loss of the ability to accumulate vital dyes in the vacuolar solution, and second by dispersion of the vacuolar solution, originally located in a central body, toward the periphery of the swelling cell

This results, in such cells, in diffuse staining with vital dyes, with a relatively high concentration of the dye at the periphery of each cell

The use of appropriate reagents showed that lipids are displaced from cells undergoing lysis under the influence of penicillin and that the liberated lipids are hydrolyzed into fatty acids

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MICROBIC DECOMPOSITION OF PANTOTHENIC ACID[1]

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The role of vitamins in the nutrition of microorganisms is well known   In many cases the accessory growth factor functions as a coenzyme, apparently being little affected itself in the process   In direct contrast to this function is the fact that at least some of the vitamins may be attacked and decomposed (either partially or completely) by certain microorganisms   In this case the vitamin serves as a substrate for the particular enzymes of the cell involved, the resulting decomposition yielding energy and materials for possible use by the cell in its various metabolic activities   Relatively little is known regarding the dissimilation of accessory growth factors, but that such can occur is not surprising in view of the wide variety of organic compounds that are subject to the action of microorganisms   Certainly, such information would lead to a better understanding of the metabolism of these vital substances, especially their biosynthesis   The literature on this subject has been reviewed by Koser and Baird (1944)

The present work was undertaken with the thought that information concerning the metabolism of pantothenic acid could be gained if it were possible to find microorganisms capable of decomposing this substance   Such microorganisms were found in soil and air (belonging to the genus *Pseudomonas*), and their action on pantothenic acid is described

EXPERIMENTAL PROCEDURES

Media and methods   The experimental work resolved itself into two main portions, one being concerned with the decomposition of pantothenic acid in cultures of growing organisms and the other with decomposition of this substance by resting or washed cells   In the former, a simple medium containing pantothenate as the only carbon source was used extensively   This medium, containing the basal salt mixture of Koser and Baird (1944), had the following composition

$(NH_4)_2HPO_4$	2 0 g
KH_2PO_4	1 5 g
NaCl	5 0 g
$MgSO_4$	0 1 g
Pantothenate	0 1 to 1 0 g*
Distilled H_2O	1,000 ml

* Calcium pantothenate was commonly used in a concentration of 0 01 per cent because of the increasingly heavy precipitate formed with larger amounts   Sodium pantothenate[3] was used in a concentration of 0 1 per cent

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The pH of the medium was adjusted to 7 5 to 7 8 and autoclaved at 15 pounds' pressure for 10 minutes   This medium will be referred to hereafter as pantothenate broth, the corresponding agar (pantothenate agar) being prepared by adding 1 5 per cent agar to the broth

Results involving turbidimetric measurements were obtained with a lumetron model 400 G photoelectric colorimeter (wave length 530 mμ)

Manometric experiments were carried out in the conventional manner, using the Warburg technique   Duplicate vessels, maintained at 30 C during experiments, were used in all cases, and to each were added 1 0 ml of M/20 phosphate buffer at pH 7 7, 1 0 ml of washed cells, and 0 5 ml of the substrate (in the side arm), in addition to KOH or H_2SO_4

Resting cells were obtained by growing the organisms on 0 1 per cent pantothenate plus 0 2 per cent asparagine agar (asparagine increased the yield of cells while not affecting pantothenate utilization) in Kollé bottles for 48 hours at 33 C   The resultant growth was then washed from the agar with M/60 buffer (in most cases a phosphate buffer at pH 7 7), and the suspension was filtered through a thin layer of glass wool and centrifuged   After a second centrifugation in graduated centrifuge tubes, the packed cells were diluted 1 30 with the M/60 buffer, and this suspension was standardized for each experiment in the lumetron colorimeter   The various substrate solutions were carefully prepared and kept in the frozen state until used

Isolation and identification of organisms   Various samples of soil were assayed for their content of pantothenate-utilizing organisms by means of two enrichment methods   The methods were essentially the same, except in one case pantothenate was added, at intervals, to moist soil, and in the other a small amount of soil was added to pantothenate broth   In both cases, repeated subcultures in pantothenate broth, combined with platings on pantothenate agar, tended to eliminate nonutilizing organisms, at the same time yielding a total of six pure cultures of bacteria that were capable of continued growth in pantothenate broth   These organisms were considered to be pantothenate utilizers (they showed little or no growth in the same medium without pantothenate) and were designated, according to their isolation numbers, as cultures 135, 401, 512, 513, 701, and 702   Three additional bacteria that could utilize pantothenate were obtained by allowing flasks of pantothenate broth to stand open in a laboratory room for several days   These were designated as cultures 2, 8, and 11

All nine cultures were periodically checked for purity, were transferred weekly in pantothenate broth and onto pantothenate agar slants, and were kept at 33 C at all times   Reserve supplies of active and dried cultures were also maintained

Attempts to isolate thermophilic pantothenate-utilizing organisms failed

Partial identification of these organisms was accomplished   All of the cultures were strikingly alike in all of their characteristics   they were gram-negative, aerobic, motile, nonsporeforming, nonpigmented, nongranular, short to medium rods, they utilized glucose, sucrose, maltose, lactose, and mannite without acid

or gas, they were indole-negative and methyl-red- and Voges-Proskauer-negative, they were non-gelatin-liquefying, they reduced litmus milk with an alkaline reaction, they produced H₂S in small amounts, and six cultures reduced nitrate (five to nitrites and one to free nitrogen) Nutrient agar plate colonies were smooth, shiny, and grayish in color   These characteristics tend to place these organisms in the family *Pseudomonadaceae* (see references by Koser and Baird, 1944, Momas, 1928, Bergey *et al*, 1939)   Flagellar stains of cultures 11 and 512, with which most of the experimental work was done, showed that both had polar flagella only (Leifson's BBL flagellar stain)   These organisms, therefore, have

TABLE 1

Effect of temperature on growth of Pseudomonas sp

CULTURE	AGE	BACTERIAL COUNT, PER ML		
		0 01% Ca pantothenate broth		Salt mixture, no pantothenate
		25 C	33 C	33 C
	hours			
11	0	100,000	80,000	15,000
	24	920,000	3,200,000	20,000
	48	23,000,000	19,600,000	35,000
	72	491,000,000	403,000,000	43,000
	96	500,000,000	480,000,000	50,000
	144	450,000,000	325,000,000	47,000
512	0	207,000	312,000	50,000
	24	1,980,000	950,000	65,000
	48	42,600,000	75,000,000	108,000
	72	520,000,000	606,000,000	121,000
	96	783,000,000	537,000,000	135,000
	144	700,000,000	480,000,000	134,000

been designated as *Pseudomonas* sp , further classification being deemed not only unnecessary but unwise

RESULTS OF EXPERIMENTS WITH GROWING CELLS

Growth in pantothenate broth   The effect of certain factors on the growth of *Pseudomonas* sp in pantothenate broth was determined, mainly to arrive at optimum conditions for later work

From table 1 showing the nutrient agar plate counts obtained with two typical cultures, it is obvious that temperature did not have much influence on growth in calcium pantothenate broth in the range of 25 to 33 C   Also, a prolonged lag phase was exhibited in this medium, rapid multiplication occurring only after 24 hours and continuing up to 72 hours   Little or no growth took place in the basal salt mixture without pantothenate, indicating that pantothenate was in fact being utilized for growth purposes   The low zero hour count in this medium was due to the fact that these tubes were inoculated from a subculture of the same medium to avoid carrying over pantothenate

The influence of pH on growth was determined by adjusting calcium pantothenate broth to various pH values (5 2, 5 5, 6 0, 6 5, 6 8, 7 2, 7 5, 7 7, and 8 0) with 0 5N HCl and 0 5N NaOH, the final concentration of pantothenate being 0 01 per cent in a total volume of 8 0 ml of medium   The incubation temperature was 33 C, and growth was determined every 24 hours with the lumetron colorimeter   The inoculum per experimental tube, which was the same in other experiments unless otherwise stated, was 0 1 ml of a 72-hour pantothenate broth culture incubated at 33 C   The results showed that all of the cultures responded similarly to variations in pH, the optimum for each being approximately 7 7   A quite rapid fall in turbidity occurred in the less alkaline media

TABLE 2

Effect of pantothenate concentration on growth of Pseudomonas sp

CULTURE	CONCENTRATION OF PANTO-THENATE	TURBIDITY READINGS	
	μg/ml	3 days	7 days
11	0	98 5	97
	100	70	67
	500	63	61
	1,000	56	52
	5,000	58	54
	10,000	60	54
	100,000	73	63
512	0	96	94
	100	75	73
	500	67	65
	1,000	60	59
	5,000	63	60
	10,000	68	63
	100,000	79	66

(no growth took place below pH 5 5), and a slight decrease was observed also at pH 8 0   After 168 hours the pH of each medium was within 0 1 to 0 2 of a point of uninoculated controls, indicating that this factor (i e , a change in pH during growth) would have no effect on growth

To determine the optimum concentration of pantothenate for growth, sodium pantothenate in amounts of 0, 100, 500, 1,000, 5,000, 10,000, and 100,000 μg per ml was added to the basal salt mixture, and turbidity readings were made after 3 days' incubation at 33 C and again after 7 days   It was found that 1,000 μg per ml was optimum for both of the cultures studied, although 5,000 μg per ml was practically as effective (table 2)   Larger amounts of pantothenate, especially 100,000 μg per ml, were definitely inhibitory to growth, although not completely so

Another experiment showed that growth in pantothenate broth was the same whether the pantothenate was added to the medium before autoclaving or

whether a filtered solution of pantothenate was added aseptically to the auto-claved basal salt mixture   The addition of certain inorganic salts ($ZnCl_2$, $CaCl_2$, $MnCl_2$, and $FeSO_4$) did not improve nor impair growth in pantothenate broth

Two analogues of pantothenic acid, pantoyltaurine[4] and dl-N-pantoyl-n-butylamine,[5] were studied for their effect on the growth of *Pseudomonas* sp in pantothenate broth   It was found that the former could support growth when used in a concentration of 10,000 μg per ml in the absence of pantothenate but not to the same extent as did the same concentration of pantothenate in the absence of pantoyltaurine   When both substances were present in the same medium, growth was considerably less than that in a medium which contained only pantothenate, unless the concentration of pantothenate was equal to or greater than the concentration of pantoyltaurine   Use of the same procedure with dl-N-pantoyl-n-butylamine, revealed that this substance (in a concentration of 10,000 μg per ml) did not support growth, nor did added pantothenate cause growth unless an excess was present   With the reverse procedure (i e , varying the concentration of the analogue and holding the pantothenate level constant) essentially the same relationship between these substances was noted   It is obvious that these results demonstrate a competitive type of inhibition

Growth in modified pantothenate broth   To determine whether the components of pantothenic acid could be utilized for the growth of *Pseudomonas* sp , various media were made up as follows β-alanine broth (40 μg and 300 μg per ml), α-hydroxy-β,β-dimethyl-γ-butyrolactone[6] broth (60 μg and 300 μg per ml), α,γ-dihydroxy-β,β-dimethyl-butyric acid (pantoic acid) broth (60 μg and 300 μg per ml), and various combinations of these media   Pantoic acid was prepared from the lactone according to the method of Sarett and Cheldelin (1945)   These media were prepared as usual except that pantothenate was replaced by the compound or compounds under study in the concentrations indicated   All nine cultures were used in these tests, and the results were obtained by visual observations of turbidity   It was found that both β-alanine and pantoic acid supported growth, the amount of growth increasing in each case as the concentration of the substrate was increased   When combined, these substances gave growth approximately equal to that in control tubes of pantothenate broth   In no instance did the lactone support growth of any of the cultures, nor did it increase the effectiveness of β-alanine broth   It seems obvious that these organisms were unable to break the lactone ring, but when this structure is ruptured (as in pantoic acid) the compound could be utilized

In another experiment, it was found that growth in pantothenate broth was not affected by removing $(NH_4)_2HPO_4$ from the basal salt mixture   By also excluding atmospheric nitrogen, it was apparent that these organisms could

deaminate β-alanine as a source of nitrogen   The salt was retained, however, as an extra nitrogen supply

The effect of added nutrients upon the growth of *Pseudomonas* sp in pantothenate broth was studied in order to try to improve the yield of bacteria for future work involving washed cells   Difco asparagine (0 2, 0 4, and 0 6 per cent) and smaco acid-hydrolyzed casein (0 5 per cent) each greatly improved growth of five of the cultures tested, as determined by visual turbidity   As was shown next, however, casein hydrolyzate "spared" the pantothenate, to a large extent, from being acted upon, whereas asparagine did not

Destruction of pantothenate during bacterial growth   In order to prove that pantothenate was actually being decomposed during growth of these *Pseudomonas* cultures and to determine the rate of this decomposition, microbiological assays for pantothenate were carried out by the method of Skeggs and Wright (1944)   With 0 1 per cent sodium pantothenate broth, it was found that all nine of the cultures had destroyed 100 per cent of the substrate within a growth period of 72 hours at 33 C   After 24 hours, cultures 11 and 512 (the only ones tested) had destroyed 20 per cent of the pantothenate

To determine the effect of added nutrients upon the destruction of pantothenate, 0 2 per cent asparagine and 0 5 per cent casein hydrolyzate were added, respectively, to pantothenate broth   The media were inoculated with cultures 11 and 512, and the contents of each were assayed for their pantothenate concentration after 24 and 72 hours' incubation at 33 C   The results with asparagine were the same as those obtained in its absence, but with casein hydrolyzate no breakdown was detected with either culture at 24 hours, and at 72 hours less than 50 per cent decomposition of pantothenate had occurred   Therefore, the use of casein hydrolyzate was discontinued, but asparagine was later incorporated in pantothenate agar for the production of washed cells

Survey of other microorganisms for pantothenate utilization   It was of interest to determine whether various representative stock cultures of bacteria or fungi possessed any native ability to decompose pantothenic acid   The methods used seemingly afforded the microorganisms optimum conditions for attacking pantothenate, but in all cases the results were negative   Therefore, for brevity, the details of this work will not be described   It is reasonable to assume, however, that in nature many of these same microorganisms may play an active role in decomposing pantothenate, having lost this function on repeated transfer in the laboratory

RESULTS OF EXPERIMENTS WITH RESTING CELLS

Deamination and Thunberg studies   An investigation into the deamination of pantothenate was performed by adding 1 0 ml of washed cells (preparation previously explained), 1 0 ml of M/20 phosphate buffer at pH 7 5, and 3 0 ml of M/50 substrate to duplicate test tubes and determining ammonia production with Nessler's reagent after incubation periods of 2, 6, and 24 hours at 33 C   It was found that pantothenate was deaminated and that the reaction progressively increased from 2 to 24 hours, meaning that the β-alanine portion

of pantothenic acid was actually the substance being acted upon   A similar experiment with β-alanine did, in fact, give the same results as were obtained with pantothenate   Also, similar results were obtained with *dl*-alanine, this substance being included to determine the specificity of the deaminase   These findings are in agreement with other reports (Stephenson, 1939) that pseudomonae can deaminate various amino acids

Preliminary to carrying out manometric experiments, it was of interest to determine whether a representative culture, culture 11, could reduce methylene blue with β-alanine, pantoic acid, pantoyl-lactone, and pantothenate as substrates   The usual Thunberg technique was used, in which 1 0 ml of washed
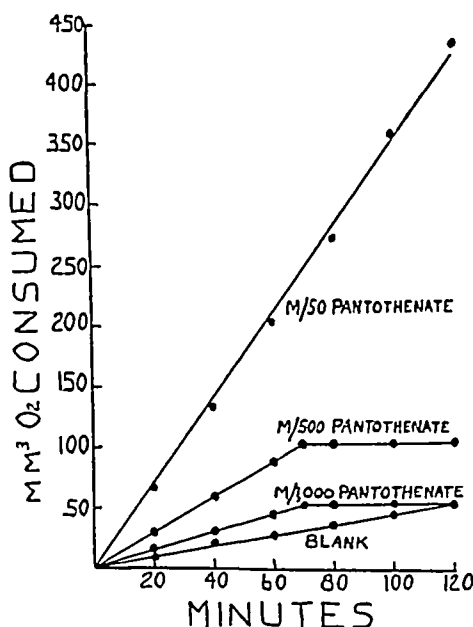
FIG 1 THE OXIDATION OF PANTOTHENATE BY PSEUDOMONAS SP , CULTURE 11

cells, 1 0 ml of M/20 phosphate buffer at pH 7 5, 0 5 ml of 1 10,000 methylene blue, and 0 5 ml of M/50 substrate were placed in duplicate Thunberg tubes, the tubes evacuated, the cells tipped in from the side arm at the zero time, and the tubes incubated at 33 C and observed visually at 5-minute intervals for decolorization   It was found that the methylene blue was completely decolorized in 65 minutes with pantothenate as the substrate, in 80 minutes with β-alanine, and in 75 minutes with pantoic acid   Tubes containing the lactone remained blue for a much longer period of time, decolorizing at the same rate as the controls

Warburg studies, oxidation of pantothenate   From the foregoing experiment, it was expected that pantothenate would be readily oxidized in the Warburg apparatus   This was found to be true, and the oxidation of various concentrations of pantothenate by culture 11 is shown in figure 1   It is apparent that
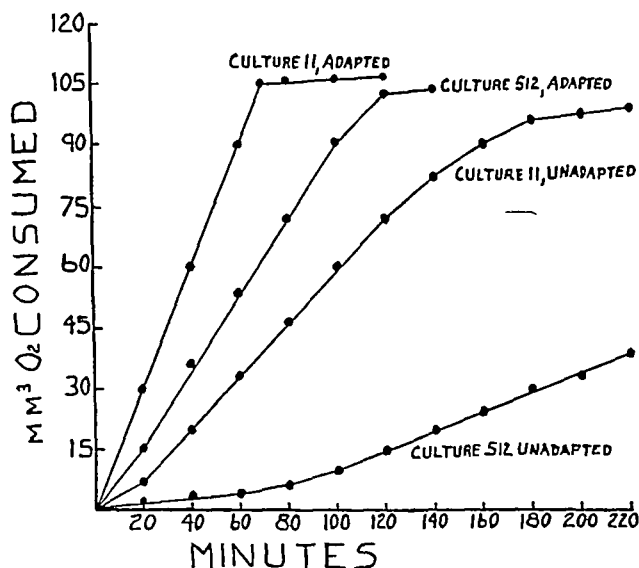
FIG 2 THE OXIDATION OF M/500 PANTOTHENATE BY ADAPTED AND UNADAPTED CELLS OF
PSEUDOMONAS SP
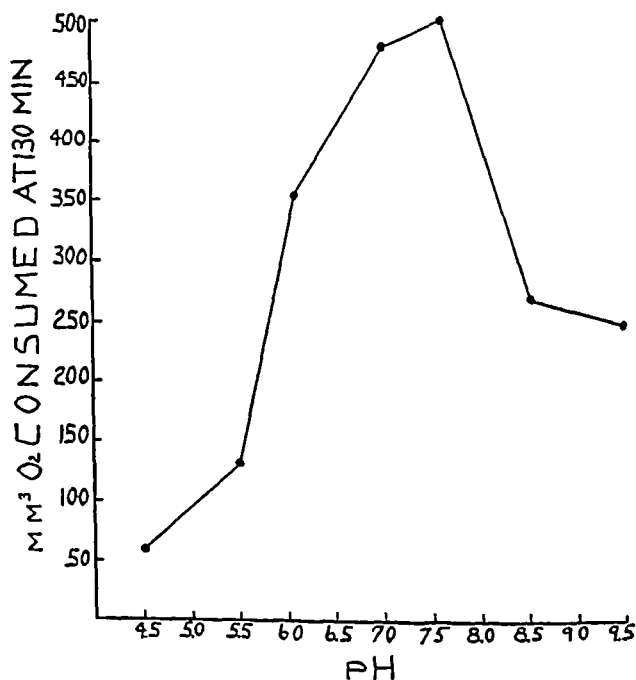
FIG 3 THE EFFECT OF pH ON THE OXIDATION OF M/50 PANTOTHENATE BY PSEUDOMONAS
SP , CULTURE 11

the reaction with M/500 and M/1,000 substrates reaches completion in approxi-
mately 70 minutes, at which time about 50 per cent of the total oxygen (theo-

retical) needed for complete combustion has been used   Although carbon dioxide curves were not established, it was found that after 70 minutes a total of 90 mm³ of this gas had been evolved from M/500 pantothenate, this being equivalent to 4 molecules of carbon dioxide   Since the oxygen consumption of this substrate was equal to 5 molecules, the respiratory quotient (R Q) at 50 per cent oxidation was 0 80 as against a theoretical R Q of 0 90 for complete combustion

Cultures 512 and 702 required 120 and 200 minutes, respectively, to achieve 50 per cent oxidation of M/500 pantothenate, but it is noteworthy that they, too, eventually completed the reaction at the same end point as did culture 11   Of the three, only culture 702 exhibited any significant lag period at the beginning of the reaction   The endogenous respiration, especially of culture 11, was rather   high, but attempts to lower it, such as aerating the cells for 1 hour prior to use, were not successful   In all cases corrections for the blank were made since it has not been definitely established that endogenous respiration is suppressed in the presence of a readily utilizable substrate, although such may be the case

The adaptive nature (Dubos, 1940) of the enzymes involved was shown by the fact that 48-hour nutrient agar cultures (unadapted) were much less active than were adapted cultures which had been maintained on pantothenate agar (figure 2)   Three transfers of the unadapted cultures on pantothenate-asparagine agar, however, fully adapted them for the utilization of pantothenate

A study into some of the factors which might influence the oxidation of pantothenate revealed that the optimum pH was around 7 7 (figure 3)   M/50 substrate was used in this experiment so that any differences in activity at the various pH levels would be magnified   An intensive study into the effect of temperature on the oxidation of pantothenate was not made, but it was found that an increase to 38 C (all experiments were conducted at 30 C) neither affected the rate nor the degree of the reaction   It was also found that the enzymes involved in the oxidation were stable for at least 10 days when stored (in the form of packed cells) in the refrigerator (not frozen)   Thus, washed cells were usually prepared a day before use and stored in the packed state overnight in the refrigerator   Also, it was revealed that physiologically young cells (48 hours) were much more active than 96-hour (or older) cells   This was expected but, unfortunately, owing to insufficient yield, 24-hour cells could not be used

Various attempts to explain the incomplete oxidation of pantothenate were unsuccessful and, without going into the details of this work, it may be said that the only logical explanation for this phenomenon was that a reaction of oxidative assimilation was occurring, the nature of which will be briefly discussed later

A survey of four nutrient agar stock cultures of bacteria, namely *Escherichia coli-communior*, *Proteus vulgaris*, *Acetobacter suboxydans, and Pseudomonas aeruginosa* showed that the first three had absolutely no activity on M/50 pantothenate, and *P   aeruginosa* had only a very minimal activity, which was not increased by three transfers on pantothenate-asparagine agar

Experiments with pantothenate analogues showed that M/50 *dl*-N-pantoyl-*n*-

oxidation of these substances may be represented by the following balanced equations

$$C_3H_7O_2N + 2O_2 \qquad = 2CO_2 + H_2O + NH_3 + (CH_2O) \qquad (1)$$
β-alanine

$$C_6H_{12}O_4 + 3O_2 \qquad = 2CO_2 + 2H_2O + 4(CH_2O) \qquad (2)$$
pantoic acid

$$C_9H_{17}O_5N + 5O_2 + H_2O = 4CO_2 + 3H_2O + NH_3 + 5(CH_2O) \quad (1 + 2)$$
pantothenic acid
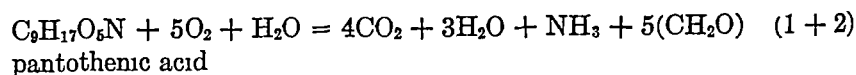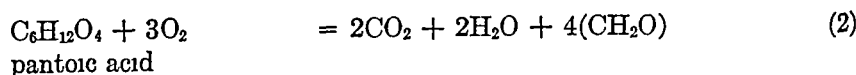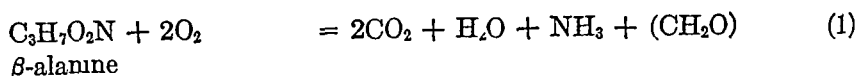
Attempts to show a 100 per cent decomposition of pantothenate with other enzyme inhibitors were unsuccessful Monoiodoacetic acid (M/5,000 and M/50,000) greatly inhibited the oxidation of M/500 pantothenate, whereas sodium azide gave results very similar to those obtained with KCN, but in different concentrations Endogenous respiration was not significantly affected by any of the inhibitors

TABLE 3

Comparative results of oxygen consumption and carbon dioxide evolution with and without potassium cyanide (M/10,000)

SUBSTRATE	OXYGEN				CARBON DIOXIDE				R Q	
	No KCN		With KCN		No KCN		With KCN		No KCN	With KCN
	mm³	mol	mm³	mol	mm³	mol	mm³	mol		
M/500 Pantothenate	110	5	175	7 8	90	4	146	6 5	0 80	0 83
M/500 β-Alanine	45	2	54	2 4	45	2	54	2 4	1 0	1 0
M/500 Pantoic acid	66	3	112	5 0	45	2	88	3 9	0 67	0 78

DISCUSSION

The results of this study again tend to emphasize the microbic decomposition of vitamins in contrast to their usual role as accessory growth factors It is striking that most of the studies of this nature have been done with pseudomonae and of significance that in each case the organisms were isolated directly from soil or other natural habitats, undoubtedly involving a process of natural adaptation What importance these studies have in relation to the decomposition of vitamins in the human intestinal tract is unknown

In the present study it was found that the lactone moiety of pantothenic acid could neither serve as a growth substrate nor as an oxidizable substrate for *Pseudomonas* sp , whereas its hydrolyzed counterpart, pantoic acid, was readily utilized by both growing and resting cells This is interesting in view of the fact that Stansly and Schlosser (1945) reported that pantoic acid is more readily utilized than is the lactone for the synthesis of pantothenic acid by *Escherichia coli* They stated that pantoic acid is the probable precursor in the biological synthesis of pantothenic acid, rather than pantolactone

Although the evidence points undeniably to a process of oxidative assimilation in the decomposition of pantothenate, it is true that a carbohydrate has not been actually demonstrated   Giesberger (1936), in similar experiments, did show an increase in the volutin content of *Spirillum serpens*, but most investigators have not studied this particular problem in detail   Suffice it to say that, knowing all of the facts, no other logical explanation of the phenomenon is possible

It is realized that actively proliferating cells may not act in the same manner on pantothenate as do resting cells, but no attempt was made here to determine this relationship   Whelton and Doudoroff (1945), however, did show that both types of cells of *Pseudomonas saccharophila* assimilated some substrates in essentially the same manner and other substrates in quite a different manner

From the results obtained here, it appears that the carbohydrate substance produced during the oxidation of pantothenate is formed with great economy by the bacterial cells, approximately 55 per cent of the carbon of the substrate being assimilated   In similar studies, Barker (1936) found that the alga, *Prototheca zopfi*, converts from 50 per cent to more than 80 per cent of the carbon of various substrates into a carbohydrate material   This undoubtedly explains the ability of some of these microorganisms to survive and multiply in simple media

The manner in which bacterial cells form this carbohydrate from a substance such as pantothenic acid is not clearly evident, although Clifton and Logan (1939) have postulated a theory, from known facts, for the formation of carbohydrate from various substrates by cells of *Escherichia coli*   Presumably, the process represents more than a mere reduction of carbon dioxide   Equally intriguing is the manner in which a poison like KCN selectively blocks the assimilatory process

SUMMARY

Bacteria of the genus *Pseudomonas* have been isolated from soil and air that could utilize pantothenate as a growth substrate in a medium containing only pantothenate and inorganic salts   β-Alanine and pantoic acid also were utilized when substituted for pantothenate, but pantoyl-lactone did not support growth

During their growth, these bacteria decomposed 20 per cent of the pantothenate in 0 1 per cent pantothenate broth within 24 hours and 100 per cent of the substrate within 72 hours

Pantoyltaurine and *dl*-N-pantoyl-*n*-butylamine, analogues of pantothenic acid, exhibited a competitive type of inhibition with pantothenate but only the former was able to support growth when substituted for pantothenate, and then to a lesser extent

Manometric studies showed that pantothenate, β-alanine, and pantoic acid were oxidized, respectively, to 50 per cent, 67 per cent, and 43 per cent of completion by a process of oxidative assimilation   Pantoyl-lactone was not oxidized

Potassium cyanide and sodium azide, in critical concentrations, caused the

oxidation of pantothenate, β-alanine, and pantoic acid more nearly to reach completion, presumably by inhibiting the processes of assimilation

dl-N-Pantoyl-n-butylamine was not oxidized and pantoyltaurine was only slightly oxidized in the Warburg apparatus

Various stock cultures of bacteria and fungi were not able to utilize pantothenate as a carbon source, nor were they able to oxidize this substance

The significance of these findings is discussed

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PRODUCTION OF MOLD AMYLASES IN SUBMERGED CULTURE

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During recent years many new and extended uses have been developed for fungal enzymes   Prominent among these are applications in the fields of food manufacturing, textile processing, and in the manufacture of malt beverages and industrial alcohol

Although certain microorganisms are capable of elaborating amylases when grown under submerged conditions either aerobically (Waldmann, 1942) or anaerobically (Hockenhull and Herbert, 1945), industrial production methods generally involve cultivation on the surface of unagitated liquid or semisolid substrates   Exceptions are the "amylo" process (Owen, 1933) and a modified amylo process (Erb and Hildebrandt, 1946), in which selected strains of *Rhizopus* or *Mucor* are grown under submerged, aerobic conditions to saccharify grain mashes prior to alcoholic fermentation   More commonly, as in the production of mold bran (Underkofler, *et al* , 1939, Boyer and Underkofler, 1945) and bacterial amylases (Beckord *et al* , 1945, 1946), media are incubated in shallow layers in closed vessels or in open trays   Attempts to adapt these microorganisms to deep tank conditions to produce comparable yields of amylase have been unsuccessful

The submerged culture method of producing amylases would have definite advantages when the product could be employed directly without concentration or purification as, for example, in the alcoholic fermentation of grain and in the manufacture of sugars and dextrins from starch   With these applications in mind a survey was made of a large number of molds to determine their ability to synthesize starch-hydrolyzing enzymes when cultured under submerged conditions   The present report deals with (1) the results of this survey of fungi, (2) the factors affecting the elaboration of amylases by promising strains, and (3) the substitution of mold amylase thus produced for distillers' malt   Pilot plant studies have been conducted with some of the promising strains disclosed herein, and the results of these experiments will be reported at a later date

METHODS

Culture survey   The cultures investigated were selected from the culture collection of the Northern Regional Research Laboratory   The basal medium for the survey of cultures was thin stillage obtained from the alcoholic fermentation

of corn and sorghums   It contained 4 to 5 per cent of dry substance, approximately one-third of which was protein (N × 6 25)   To favor the growth of all the organisms studied, 2 per cent of glucose and 0 5 per cent of calcium carbonate were added to the stillage   This medium was sterilized with steam at 20 p s i gauge for 30 minutes

For evaluation of the selected organisms for amylase production, cultures were grown first in 50 ml of thin stillage medium contained in 200-ml flasks   After 24 hours' incubation, 10 ml of culture were transferred to 200 ml of the supplemented stillage medium contained in 1-liter flasks   All cultures were incubated at 30 C and were shaken continuously at 90 three-inch strokes per minute in a Kahn type shaker   Samples were removed periodically for the determination of amylase activity

Culture liquors were analyzed for the presence of dextrinizing enzyme by the method of Sandstedt et al (1939) as modified by Olson, Evans, and Dickson (1947)   Units of dextrinizing enzyme reported herein are the grams of soluble starch (Merck, Lintner) which, in the presence of excess beta-amylase, are dextrinized in 1 hour at 20 C [4]

Variations of cultural conditions   To determine the influence of different carbohydrate and protein sources on amylase production, a fungal strain which was found to possess exceptional amylolytic activity was grown under conditions identical to those obtaining in the survey except that various protein and carbohydrate materials were substituted for thin stillage and glucose

A study of the effects of different concentrations of calcium carbonate and calcium chloride was made under the same cultural conditions used in the survey except that 2 per cent of ground corn was substituted for 2 per cent of glucose

Aeration rate studies were conducted on a somewhat larger scale than were the aforementioned experiments, that is, 4-liter quantities of stillage medium supplemented with 2 per cent of corn meal and 0 5 per cent of calcium carbonate were dispensed in 8-liter pyrex cylinders equipped with lids of aluminum plate and with air spargers of perforated aluminum tubing   These were sterilized with steam at a pressure of 25 p s i gauge for 1 hour, cooled, and inoculated with 5 per cent by volume of a 24-hour culture   Air for use in the experiments was filtered through sterile cotton before introduction into the medium

It was observed in the course of these experiments that strains which saccharified starch rapidly, and consequently were most suitable as replacements for barley malt, formed appreciable amounts of maltase   Following this observation, both dextrinizing and maltase activities of culture liquors were determined   Maltase activity was measured by determining the increase in reducing power by the method of Somogyi (1945) after incubating 10 ml of culture filtrate with 20 ml of a 1 05 per cent solution of maltose for 2 hours at 30 C   The enzyme-substrate mixture was maintained at a pH of 4 6 by the addition of acetate buffer to the maltose solution

[4] A recent collaborative study of the *alpha* amylase values of experimentally produced barley malts in which this method was used showed a range of activity from 9 8 to 30 5 units per gram   Commercial distillers' malt contains in the neighborhood of 25 units per gram

Conversion and fermentation of grain mashes   Cultures producing appreciable quantities of amylase were further evaluated by determining their ability to replace barley malt in the alcoholic fermentation of corn   Forty-nine and one-half g of ground corn and 0 5 g of ground barley malt were placed in 500-ml Erlenmeyer flasks, and 170 ml of tap water heated to 70 C were added   The flasks were placed in a 70 C water bath and the grain slurries were stirred intermittently for 30 minutes   The mashes thus premalted were then cooked in the autoclave at a steam pressure of 25 pounds for 30 minutes and cooled to 75 C, mold culture liquor was added, together with sufficient water to lower the temperature to 55 to 56 C   Conversion was continued at 55 to 56 C for 30 minutes, during which period the mashes were agitated frequently   The same procedure was followed with the control mashes saccharified with malt except that 45 g of corn and 5 g of barley malt were used, 0 5 g of malt again being used for premalting and 4 5 g for conversion   Converted mashes were cooled to 30 C and inoculated with 2 per cent by volume of a 24-hour culture of distillers' yeast, strain NRRL Y567   The final volume in each flask was approximately 250 ml Fermentation was conducted at 30 C for 72 hours, during which time the flasks were weighed periodically   The beers were then brought to a volume of 300 ml and aliquots of 200 ml taken for the determination of alcohol   One hundred ml of distillate were collected from each aliquot, and the concentration of alcohol in the distillate was determined by measuring its refractive index

EXPERIMENTAL RESULTS

The results of the survey of fungi for ability to produce amylase in supplemented thin stillage medium are presented in table 1   Of 80 cultures of *Penicillium*, representing 18 species, only 8 formed detectable quantities of dextrinizing enzyme   All of these were of relatively low activity, the best being a strain of *P purpurogenum* which gave 0 6 units per ml

Two hundred seventy-eight of the cultures that were studied belonged to the genus *Aspergillus* and represented 41 different species   Only 34 members of this group elaborated dextrinizing enzyme   The culture liquors from active organisms varied from 0 1 to 15 3 units per ml   Although there was considerable variation between strains within a species, a high percentage of strains of *A oryzae, A wentii,* and *A niger* was active   *Aspergillus niger* NRRL 337 gave the highest potencies of any organisms tested in the survey   Under the most favorable conditions, potencies up to 22 5 units per ml were obtained with it   On a dry basis (culture liquors contained about 2 per cent of solids) such preparations would have a potency of 1,125 units per gram

Subsequently, *Aspergillus niger* strains NRRL 326, 330, and 679 were found to elaborate an enzyme complex which rapidly saccharified starch, although the formation of dextrinizing enzyme was not so marked as with *A niger* NRRL 337

When strains of *Rhizopus, Mucor,* and *Monilia* were grown under the same conditions, little or no dextrinizing enzyme was produced, although excellent growth was obtained   Despite their apparent lack of dextrinizing enzyme, culture liquors from a strain of *Rhizopus* NRRL 1891 received under the label "Rhizopus 'Boulard,' " were capable of considerable saccharification of grain

TABLE 1

The production of amylase by various fungi grown submerged in thin stillage medium

GENUS	CULTURES TESTED	NUMBER ACTIVE*	ACTIVE CULTURES		CONCENTRATION OF DEXTRINIZING ENZYME PRODUCED
			species	*NRRL no*	*units/ml*
Penicillium	80	8	P urticae	991	0 1
			P roseo citreum	889	0 1
			P spiculosporum	1027	0 2
			P chlorophaeum	816	0 2
			P citreo-roseum	835	0 2
			P aurantio-griseum	972	0 2
			P brunneo-rubrum	842	0 3
			P purpurogenum	1064	0 6
Aspergillus	278	34	A versicolor	231	0 1
			A candidus	305	0 6
			A alliaceus	315	1 7
			A foetidus	341	1 3
			A niger	622	0 1
			A niger	624	0 1
			A niger	606	0 1
			A niger	617	0 1
			A niger	607	0 1
			A niger	605	0 1
			A niger	614	0 1
			A niger	354	0 4
			A niger	679	1 1
			A niger	326	2 2
			A niger	330	6 0
			A niger	337	15 3
			A niger	363	1 6
			A wentii	382	0 1
			A wentii	378	0 1
			A wentii	1207	0 2
			A wentii	381	0 3
			A wentii	1778	0 4
			A wentii	377	0 4
			A wentii	1269	0 6
			A oryzae	480	0 1
			A oryzae	474	0 1
			A oryzae	464	1 2
			A oryzae	449	2 9
			A oryzae	694	3 1
			A oryzae	698	3 3
			A oryzae	454	3 0
			A flavus	488	0 1
			A flavus	491	0 2
			A gymnosardae	505	0 2
Rhizopus	5	none			
Mucor	3	none			
Monilia	1	none			

* Cultures were termed active if the α amylase activity obtained in their filtrates equaled or exceeded 0 1 unit per ml

mashes (table 6)   Other species of *Rhizopus* and *Mucor* were similar in behavior, suggesting that these organisms have amylolytic enzyme systems different from barley malt and the other molds examined in this study   In contrast to this observation, Leopold and Starbanow (1943) have reported the production of both α- and β-type amylolytic enzymes by *R japonicus*

TABLE 2

The production of dextrinizing enzyme by Aspergillus niger NRRL 337 cultivated in various media

PROTEIN SOURCE	CARBOHYDRATE SOURCE	CONCENTRATION OF DEXTRINIZING ENZYME
		units/ml
Corn steep liquor, 3%	None	2 2
Corn steep liquor, 3%	Glucose, 2%	8 2
Corn steep liquor, 3%	Molasses, 2%	4 6
Corn steep liquor, 3%	Corn meal, 2%	10 2
Dried tankage, 2%	None	2 1
Dried tankage, 2%	Glucose, 2%	9 3
Dried tankage, 2%	Molasses, 2%	11 5
Dried tankage, 2%	Corn meal, 2%	8 7
Soybean meal, 2%	None	7 9
Soybean meal, 2%	Glucose, 2%	7 4
Soybean meal, 2%	Molasses, 2%	8 5
Soybean meal, 2%	Corn meal, 2%	11 2
Thin stillage	None	1 7
Thin stillage	Glucose, 2%	11 5
Thin stillage	Molasses, 2%	7 9
Thin stillage	Corn meal, 2%	16 5
Thin stillage	Xylose, 2%	5 3
Thin stillage	Lactose, 2%	6 7
Thin stillage	Sucrose, 2%	11 0
Thin stillage	Maltose, 2%	14 5

Enzyme determinations were made after cultures were shaken for 5 days
Composition of medium   Protein and carbohydrate as shown plus 0 5 per cent calcium carbonate

Factors affecting enzyme production by Aspergillus niger NRRL 337   To determine whether nutrients other than those present in thin stillage were satisfactory for amylase production, media containing protein from several other sources were supplemented with various carbohydrates   Calcium carbonate was added to give a concentration of 0 5 per cent   After sterilization, the media were inoculated with 2 per cent by volume of a submerged culture of *Aspergillus niger* NRRL 337 and incubated, with continuous shaking, for 5 days   The results are shown in table 2

Thin stillage, corn steep liquor, and animal tankage when not supplemented with carbohydrate gave low yields of amylase, but soybean meal appeared to be

satisfactory without added carbohydrate   When commercial glucose, molasses, or corn meal was added to the protein basal media, good amylase formation re sulted except when corn steep liquor was supplemented with molasses   In this series of experiments the highest enzyme concentration (16 5 units per ml) was obtained with thin stillage to which corn meal was added   Sucrose and maltose gave good enzyme formation, whereas xylose and lactose were less effective when added to thin stillage   These results indicate that a wide variety of carbohydrates in conjunction with proteinaceous substances of animal and plant orgin can be employed for the production of amylase by this organism

The influence of calcium carbonate on amylase production is demonstrated in the following experiment the results of which are shown in table 3   Calcium carbonate, in varying amounts to give concentrations ranging from 0 to 1 0 per

TABLE 3

The effect of calcium carbonate and calcium chloride on the production of dextrinizing enzyme by Aspergillus niger NRRL 337

SOURCE OF CALCIUM		FINAL pH	α AMYLASE
Salt added	Concentration		
	per cent		*units/ml*
None		4 0	1 5
CaCO₃	0 10	4 3	7 9
CaCO₃	0 25	4 9	8 9
CaCO₃	0 50	5 3	9 2
CaCO₃	1 00	5 4	8 5
CaCl₂	1 00	3 7	1 2

Cultures were analyzed after an incubation period of 3 days at 30 C
Composition of base medium   Distillers' thin stillage plus 2 per cent corn

cent, was added to thin stillage containing 2 per cent ground corn   *Aspergillus niger* NRRL 337 was cultured in these media for 3 days, after which the culture liquors were analyzed for dextrinizing potency   Whereas the enzyme activity was low in the absence of calcium carbonate, the addition of 0 1 per cent calcium carbonate gave more than a 5-fold increase in dextrinizing power, that is, from 1 5 to 7 9 units per ml   The optimum concentration of calcium carbonate appeared to be in the neighborhood of 0 25 to 0 5 per cent, resulting in potencies of 8 9 and 9 2 units per ml, respectively   The pH of the fermented liquors ranged from 4 0 in media without calcium carbonate to 4 3 to 5 4 in those in which it was used   When calcium chloride at a concentration of 1 per cent was substituted for calcium carbonate, the final pH was 3 7, and the enzyme production was lower than that in the control without added calcium salt   Since it is well known that α-amylase is readily inactivated at a pH of 4 2 or lower, it appears that the principal action of the calcium carbonate in stillage medium is to maintain the pH above this point during the fermentation   However, a specific stabilizing effect of the calcium ion upon mold dextrinizing amylase has been demonstrated (Nakamura, 1931), and this may have been a contributing factor in those instances in which the reaction was favorable to amylase stability

The influence of aeration upon amylase production is shown in table 4   *Aspergillus niger* NRRL 337 was grown in supplemented thin stillage medium in glass cylinders, as previously described   The aeration rate was varied from 0 25 volumes to 1 0 volume of air per volume of medium per minute   Dextrinizing enzyme and pH were determined daily from the second through the seventh day   It was found that enzyme synthesis increased progressively with increased rates of aeration   With 0 25 volume of air the final potency of the liquor was 2 4 units per ml, with 0 5 volume of air, 9 0 units per ml, and with 1 0 volume of air, 22 5 units per ml   In larger fermentations in which media were both aerated and agitated, a lower rate of aeration was found to be adequate for maximum enzyme production (Le Mense *et al*, 1947)

Substitution of mold culture liquors for malt in alcoholic fermentations   Culture liquors from the preceding experiment were investigated for their ability to replace barley malt in the saccharification of grain mashes for alcoholic fermentations   The liquors were used at levels of 8, 13, and 20 per cent of the final mash

TABLE 4

The influence of the rate of aeration on the production of dextrinizing enzyme by Aspergillus niger NRRL 337

AERATION RATE	DEXTRINIZING ENZYME AFTER					
	2 days	3 days	4 days	5 days	6 days	7 days
L air/L medium/ minute	*units/ml*					
0 25	0 6	1 4	1 8	2 7	2 2	2 4
0 5	4 8	6 9	7 9	8 2	9 0	9 0
1 0	6 2	8 9	12 9	15 8	22 0	22 5

volume   One-tenth of this amount in each instance was added as premalt   Corn was the only grain used in the mashes saccharified with mold amylase, whereas control mashes contained 90 per cent corn and 10 per cent barley malt, one-tenth of the malt also being employed as premalt   The results of these experiments are shown in table 5

Malt-converted control mashes gave an average yield of 5 15 proof gallons of alcohol per bushel of grain   When used at a level of 13 per cent of the mash volume, culture liquors produced by aerating at 0 25 volume of air per volume of medium per minute for a 7-day culture period gave only 4 24 proof gallons of alcohol per bushel of grain   Culture preparations aerated at 0 5 volume per volume of medium per minute gave yields equivalent to or better than malt when used at 13 and 20 per cent levels after 4 days of incubation and when used at 8, 13, and 20 per cent levels after 7 days of incubation   Cultures aerated at 1 volume of air per volume of medium per minute were satisfactory in all cases except those to which a liquor cultured for 2 days was added at an 8 per cent level   The highest alcohol yields, amounting to 5 50 and 5 40 proof gallons per bushel, were obtained with 20 per cent levels of culture liquor aerated at 0 5 volume of air per volume of medium per minute   When the greater quantity of

with limited maltose failed to increase either the rate or the degree of saccharification of grain mashes (*A oryzae* NRRL 694)   This is of special interest in view of the indicated correlation between α-amylase potency and yield of alcohol with distillers' malts (Thorne *et al* , 1945)   It must be assumed, therefore, either that malt α-amylase is capable of more complete breakdown of starch than is the corresponding enzyme from mold, or that other enzyme components of malt are more active in saccharification than generally believed   Mold amylases might also comprise other enzymes than the two demonstrated to be present, as manifested by the amylolytic activity of preparations from a strain of *Rhizopus* NRRL 1891 (labeled *Rhizopus* "Boulard" as received), which display limited dextrinizing potency

It may be of interest to compare dry weights and dextrinizing units obtained in mashes wherein good alcohol yields resulted with fungal amylases with corresponding figures for the barley malt control mash   Thus in the best alcoholic fermentation obtained with fungal amylases as presented in table 5 a culture liquor containing about 2 5 per cent of dry solids and supplied at the level of 20 per cent of the mash volume contributed about 1 25 g of solids and 395 dextrinizing units of α-amylase and resulted in a yield of 5 50 proof gallons of alcohol per bushel of grain   In the barley malt control fermentation 5 0 g of malt with an α-amylase activity of 24 units per g (dry basis) and a mixture content of 8 05 per cent contributed 4 6 g of dry solids and 110 dextrinizing units of α-amylase and gave a yield of 5 15 proof gallons of alcohol per bushel of grain   These figures demonstrate that good preparations of fungal amylase offer higher α-amylase activity per unit of dry weight than does barley malt and that the higher yields of alcohol were associated with greater dextrinizing activity of the mold preparation employed

The media and techniques employed in the present study might well be utilized industrially for the production of fungal amylases   By selection of the proper culture, products rich in dextrinizing or both dextrinizing and saccharifying enzymes could be obtained   A large number of substrates, now by-products or waste products of industrial grain processing, could be employed satisfactorily   Culture liquors, when feasible, could be utilized without prior treatment or the enzymes could be concentrated and recovered as dry preparations as is now done with enzymes produced by *Aspergillus oryzae* cultivated on cereal bran (mold bran)   Drying of the culture liquor after the removal of mold mycelium and suspended solids would result in products having amylase potency 40- to 50-fold greater than that in the untreated culture

ACKNOWLEDGMENT

SUMMARY

The ability of more than 350 fungi to produce amylase when grown under submerged, aerobic conditions has been determined   Cultures of *Rhizopus*

Mucor, Penicillium, Aspergillus, and *Monilia* were represented With the exception of a few species of *Aspergillus,* all of the organisms investigated elaborated only limited quantities of amylase or were incapable of its formation Among the aspergilli, substantial amounts of dextrinizing enzyme were produced by *A wentii, A oryzae,* and *A alliaceus,* whereas both dextrinizing and saccharifying enzymes were formed by a few strains of *A niger* The presence of maltase in appreciable quantities was noted among the strains which actively saccharified starch

High amylase-producing strains of *Aspergillus niger* such as NRRL 337 were readily grown on a medium composed of thin stillage supplemented with 2 per cent of corn meal and 0 5 per cent of calcium carbonate After incubation under continuous aeration 3 to 5 days, culture liquors were satisfactory replacements for distillers' malt in the alcoholic fermentation of corn

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EFFECT OF THE COMPOSITION OF THE SPORULATION MEDIUM ON CITRIC ACID PRODUCTION BY ASPERGILLUS NIGER IN SUBMERGED CULTURE[1]

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Extensive work has been done (Foster, 1939, Perlman, Dorrell, and Johnson, 1946, Porges, 1932) on the nutritional requirements of *Aspergillus niger* and on the relation between the constitution of the fermentation medium and the yield of citric acid by this organism   However, no work has been reported in which the effect of the composition of the sporulation medium has been studied   Doelger and Prescott (1934), however, have noted that successive transfers of the culture on a synthetic medium increased citric acid yields   In the course of a study of citric acid production by submerged culture (Shu and Johnson, 1947) it was noted that the composition of the sporulation medium had a great effect on citric acid yield in fermentations in which the spores were used as inoculum   The experiments reported in the present paper were designed to determine the cause of this variation in yield

METHODS

A strain of *Aspergillus niger* from culture 72-4 (Perlman, Kita, and Peterson, 1946) was used throughout the experiments   The stock culture was carried on soil   In order to reduce the amount of soil substances carried over, cultures were carried through three successive sucrose agar slants made with medium A, shown in table 1   The second of these transfers was kept as a substock culture for the entire experiment   A water suspension of spores was made from the third transfer with 5 ml of sterile distilled water

This suspension was used to inoculate the agar medium under investigation 1 loopful for an agar slant and 0 5 ml for a bottle plate   All slants were made with 4 ml of agar medium in 18-by-150-mm pyrex test tubes   The slope of the slants was made approximately 15 degrees with respect to the axis of the tube   Bottle plates were made with 25 ml of agar medium in a 6-oz rectangular bottle   This amount gave a layer 0 5 cm thick with a 72 sq cm agar surface when the bottles were placed in a horizontal position   The media were sterilized at 120 C for 20 minutes   The inoculated slants or plates were incubated at 30 C until the entire agar surface was uniformly covered with spores

Suspensions of spores grown on experimental agar media were made with 5 ml of sterile water for slants and with 50 ml for bottle plates   Approximately 1 5 ml

of the suspension was used to inoculate 500-ml cotton-plugged Erlenmeyer flasks containing 50 ml of fermentation medium   The composition of the fermentation medium is shown as medium B in table 1   The inoculated flasks were incubated at 25 C on a shaker, rotating horizontally, describing a circle 1 inch in diameter at a speed of 270 rpm   All results reported represent the average of triplicate flasks

At intervals of 5, 7, and 10 days, samples were taken and analyses were made for residual sugar, titratable acidity, and, in some cases, citric acid   The figures given in the tables are for samples taken at 10 days except when otherwise stated Residual sugar was determined by the method of Shaffer and Somogyi (1933) and citric acid by the method of Perlman, Lardy, and Johnson (1944)   The titratable

TABLE 1

Composition of media

CONSTITUENTS	MEDIUM A	MEDIUM B	MEDIUM C
	wt/L	*wt/L*	*wt/L*
Domino sucrose	140 g	140 g	140 g
Difco agar	20 g		
KH$_2$PO$_4$	1 g	1 g	1 g
MgSO$_4$ 7H$_2$O	0 25 g	0 25 g	0 25 g
NH$_4$NO$_3$	2 5 g	2 5 g	2 5 g
HCl	to pH 4 5	to pH 3 1	to pH 2 3
Trace metals			
Cu^{++}	0 14 mg	0 06 mg	0 06 mg
Zn^{++}	1 4 mg	0 25 mg	0 25 mg
Fe^{+++}	2 2 mg	1 3 mg	0 45 mg
Mn^{++}	<1 μg	<1 μg	<1 μg

The listed quantities of metals include the amounts present as impurities in other constituents of the media

acidity was expressed in terms of anhydrous citric acid   The presence of certain trace elements was determined colorimetrically with the γ, γ' dipyridyl method for iron, dithizone for zinc, carbamate for copper, and periodate for manganese (Sandell, 1944)   The yield of citric acid was expressed as the percentage of added sugar (grams anhydrous citric acid per 100 grams added sucrose)

EXPERIMENTAL RESULTS

Effect of medium on rate of sporulation   Rectangular bottle plates were used in these experiments   The composition of the media tested was the same as medium A, table 1, except for the components which were varied   The minimum time required for the spores to cover the entire plate was used as a measure of the rate of spore formation   The results are summarized in table 2   Increasing the concentration of Zn, NH$_4$NO$_3$, and KH$_2$PO$_4$ retarded the rate of spore formation, but increasing the concentration of Mn or malt extract favored spore formation   Abundant spores were formed in 48 hours if Mn or malt extract was added

Effect of trace metals in sporulation medium on citric acid yield   Agar slants

were used in these experiments   Table 3 summarizes the results of the addition
of Fe, Cu, Zn, and Mn, and their combinations, to the basal agar sporulation
medium A   The presence of Mn at a level of 9 3 mg per liter lowered the acid
yield to 35 per cent, which is only 50 per cent of the basal medium control   Iron
added at a level of 8 mg per liter of medium also showed considerable effect

<div align="center">TABLE 2</div>

Effect of the composition of medium on rate of sporulation

CONSTITUENT VARIED IN MEDIUM A	QUANTITY PRESENT		MINIMUM TIME FOR SPORES TO COVER AGAR SURFACE
			hours
None			68
pH	6 0		68
	7 5		96
Sucrose	200	g/L	68
	50	g/L	68
KH$_2$PO$_4$	5 0	g/L	>240
	2 5	g/L	72
NH$_4$NO$_3$	5 0	g/L	>240
	0 5	g/L	52
MgSO$_4$ 7H$_2$O	0 5	g/L	68
	0 05	g/L	68
Mn^{++}	9 3	mg/L	48
Fe^{+++}	10 2	mg/L	60
	3 0	mg/L	68
Cu^{++}	3 5	mg/L	68
	0 5	mg/L	68
Zn^{++}	25 4	mg L	144
	3 8	mg/L	96
Malt extract	1 5	g/L	48

Zinc alone did not exhibit any significant effect, but it exhibited some antagonistic
effect against manganese   Copper showed a similar effect   Simultaneous addi-
tion of Cu, Zn, and Fe at levels of 0 34 mg, 2 4 mg, and 0 8 mg, respectively, per
liter of basal medium was found to give the highest acid yield in the fermentation
test   A yield of 80 per cent total acidity calculated as citric acid on added sugar
was obtained in 10 days of fermentation   About 90 per cent of this total acidity
was due to citric acid
    The stability of the culture in the medium (no 18, table 3) was tested by 18
successive spore transfers   At intervals of 6 transfers, fermentation tests were

made   The results are shown in table 4   No significant changes in acid production were observed

TABLE 3

Effect of metallic ions in sporulation media on acid production

NO	METALLIC ION ADDED TO SPORULATION MEDIUM A				YIELD OF ACID* IN METUM B ON AVAILABLE SUGAR PER CENT
	Mn	Zn	Cu	Fe	
	mg/L	mg/L	mg/L	mg/L	
1	0	0	0	0	70
2	93	0	0	0	14
3	9 3	0	0	0	35
4	1 9	0	0	0	53
5	0	24	0	0	69
6	0	2 4	0	0	71
7	0	0	3 4	0	60
8	0	0	0 34	0	66
9	0	0	0 07	0	54
10	0	0	0	8	48
11	0	0	0	0 8	53
12	0	2 4	0 34	0	63
13	9 3	2 4	0	0	45
14	0	2 4	0	0 8	70
15	9 3	0	0 34	0	45
16	9 3	0	0	0 8	37
17	0	0	0 34	0 8	56
18	0	2 4	0 34	0 8	80
19	9 3	2 4	0 34	0	46
20	9 3	2 4	0	0 8	22
21	9 3	0	0 34	0 8	20
22	9 3	2 4	0 34	0 8	54

* Titratable acidity calculated as anhydrous citric acid

TABLE 4

Effect of successive spore transfers of the culture on acid production

NUMBER OF TRANSFERS	YIELD OF ACID* ON AVAILABLE SUGAR PER CENT
0	80
6	80
12	87
18	75

* Titratable acidity calculated as anhydrous citric acid

The metals might exert the effects shown in table 3 either by being carried over by the spores into the fermentation medium, or by causing some physiological changes in the spores   Since it is known (Perlman, Dorrell, and Johnson, 1946) that the presence of appreciable quantities of manganese in the fermentation medium reduces yields of citric acid in surface fermentations, it seemed desirable

to determine the quantity of Mn added to the fermentation medium by the spore inoculum   Spores were grown in bottle agar plates containing various levels of Mn   Spore suspensions from each of the bottle plates were made with 50 ml sterile distilled water containing 10 per cent ethyl alcohol   The suspensions were filtered aseptically through glass wool into previously sterilized centrifuge tubes The tubes were then centrifuged and the supernatant was pipetted out   The spores were washed twice with 50-ml portions of distilled water and finally re-suspended in 50 ml distilled water   For each of the fermentation flasks 1 5 ml of this suspension was used as inoculum   The remaining spore suspension was used for the determination of manganese

TABLE 5

Effect on acid production of manganese carried into fermentation medium with spore inoculum

No	Mn ADDED TO SPORULATION MEDIUM A	Mn CARRIED TO FERMENTATION MEDIUM B	Mn ADDED TO FERMENTATION MEDIUM B	YIELD OF TITRATABLE ACID ON AVAILABLE SUGAR	YIELD OF CITRIC ACID[*] ON	
					Available sugar	Utilized sugar
	mg/L	*µg/L*	*µg/L*	*per cent*	*per cent*	*per cent*
1†	0	<0 02	0	66	57	64
2	0	<0 02	0 4	74	69	75
3	0	<0 02	3	50	45	56
4	0	<0 02	15	23	19	21
5†	0 93	0 4	0	68	57	66
6†	9 3	3	0	44	40	56
7†	93 0	16	0	21		

* By pentabromoacetone method
† Results of 12 days' fermentation

Another series of fermentations was prepared and inoculated with spores produced on the basal (Mn-free) medium   To these flasks were added amounts of manganese equal to those introduced to the first series of fermentation flasks with the spores grown on the Mn-containing media   The results are summarized in table 5   It may be seen that the amount of Mn carried over with the washed spore inoculum to the fermentation medium is sufficient to retard the acid production, and that as little as 3 µg Mn per liter of fermentation medium appreciably lowers the citric acid yield

Addition of malt extract   As shown in table 6, the addition of Trommer's malt extract to the sporulation medium at a level of 1 5 g per liter decreased the acid yield   The organic components of the malt extract seem to be responsible for this reduction, because the addition of the equivalent amount of the malt extract ash to the agar plate medium favored acid production in the fermentation test Furthermore, the addition of malt extract to the agar medium containing manganese at a level of 9 3 mg per liter exhibited a definite additional influence on acid production   The results are shown in table 7   This effect of Mn and malt

extract is not noticeable if the fermentation test is run by the surface culture method (table 6) with the fermentation medium C (table 1)

TABLE 6

Effect of addition of malt extract (to sporulation medium) on acid production

SUBSTANCES ADDED	QUANTITY ADDED	YIELD OF ACID PRESENT*	METHOD OF FERMENTATION
	g/L		
None		66	submerged
Trommer malt extract	1 5	55	submerged
Trommer malt extract ash	Equivalent to 1 5 g malt extract	76	submerged
Trommer malt extract	1 5	28	submerged
Mn	0 0093		
None		60	surface
Trommer malt extract	1 5	57	surface
Mn	0 0093		

* Titratable acidity calculated as anhydrous citric acid

TABLE 7

Retardation of acid production by simultaneous presence of malt extract and manganese in sporulation medium

BASAL MEDIUM A +		YIELD OF ACID ON AVAILABLE SUGAR*
Mn	Trommer malt extract	
mg/L	*g/L*	*per cent*
9 3	0 00	33
9 3	0 01	27
9 3	0 05	12
9 3	0 10	10
9 3	1 00	11

* Titratable acid calculated as anhydrous citric acid on 7 days' fermentation

SUMMARY

The addition of Mn and Trommer malt extract at a level of 9 3 mg and 1 5 g, respectively, to 1 liter of basal agar plate medium accelerated spore formation, whereas increasing the concentration of KH_2PO_4, NH_4NO_3, and Zn retarded spore formation

The presence of Mn in the sporulation medium at a level of 9 3 mg per liter retarded citric acid production in submerged fermentations in which the spores were used as inoculum   The effect is shown to be attributable to the amounts of Mn carried over into the fermentation medium by the washed spore inoculum

The presence of Mn and malt extract in the sporulation medium reduced the acid production in submerged fermentation, but not in the surface culture fermentation

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CLOSTRIDIA IN GAS GANGRENE AND LOCAL ANAEROBIC INFECTIONS DURING THE ITALIAN CAMPAIGN

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When World War II began, some of the experimental results obtained on animals indicated that sulfonamides applied locally and taken by mouth might prevent infections with anaerobes of the gas gangrene group   It became evident, even in the Western Desert and Tunisia, that anaerobic infections would occur in spite of sulfonamide prophylaxis   When the fighting took place on the more cultivated soil in Sicily and Italy, the incidence of gas gangrene increased   Antiserums made in the United States, usually containing antitoxin only for *Clostridium perfringens* (*B welchii*) and *Clostridium septicum*, did not appear to prevent gas gangrene and were of limited value in the therapy of cases (Jergesen, 1944)   The question arose as to the incidence of *Clostridium novyi* (*B oedematiens*) in anaerobic infections, it was questioned if the poor results with serum could be attributed to the lack of *C novyi* antitoxin in some American polyvalent gas gangrene antiserums

In order to determine the incidence of *C novyi*, the clostridial flora of 25 cases of gas gangrene that occurred in Italy was studied (Stock, 1944)   In a second study, made while the fighting was in the Northern Apennines, 5 additional cases of gas gangrene and 7 of local anaerobic infections were cultured, and at the same time an effort was made to determine the incidence and significance of positive blood cultures   It appeared important to learn whether therapy could save a case of gas gangrene once the causative organisms had entered the blood stream   Although only the preliminary phase of the latter study was completed, it may be of value to record these results and to summarize our entire findings because so few reports on cultures in gas gangrene or other anaerobic infection in World War II have appeared (MacLennan, 1943, 1944, MacLennan and Macfarlane, 1944, Jeffrey and Thomson, 1944, Smith and George, 1946)

MATERIALS AND METHODS

Specimens of muscle or blood were placed in chopped meat medium at surgical operation and forwarded to the laboratory   Anaerobic jars of the McIntosh-Fildes type, but without a heating coil, were fashioned from 105-mm shell cases (see Smith and George, 1946)   Two g of palladium-asbestos (Fildes, 1917) covered by a wire screen served as catalyst   Anaerobes were grown on the surface of thioglycolate blood agar plates from inoculums of unheated, heated (80 C), and enriched heated samples   Isolation and identification of clostridia were

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cultures were injected and no calcium chloride was used (Bullock and Cramer, 1919)

DISCUSSION

In general, the distribution of clostridia found in cases of gas gangrene was similar to that described in published reports (Weinberg and Seguin, 1918, Medical Research Committee, Brit , 1919, Sordelli, 1923, Zeissler and Neller, 1928, MacLennan, 1943, 1944, MacLennan and Macfarlane, 1944, Smith and George, 1946)   The presence of *C novyi* in about 50 per cent of cases of gas gangrene is confirmed   *C novyi* was found in soil by Zeissler and Rassfeld (1928) in 64 per cent of samples, so that a high incidence is possible in wounds If the prophylactic and therapeutic efficiency of gas gangrene antiserums is to be determined, it would seem necessary to include *C novyi* antitoxin in the polyvalent serum (see Hall, 1946)   Gas gangrene toxoids for immunization should contain *C novyi* toxoid as a component (Robertson and Keppie, 1943)

C septicum was isolated only once in 30 cases of gas gangrene   In a large series of cases, Weinberg and Seguin (1918) found a 13 per cent and MacLennan (1943) a 19 per cent incidence for this species   On the other hand, Zeissler and Neller (1928) isolated only 1 strain of *C septicum* from 22 cases of gas gangrene in German civilians, and Zeissler and Rassfeld (1928), in an examination of soil, found an incidence of 8 per cent

None of the strains of *C bifermentans* isolated in our studies was pathogenic for guinea pigs by the methods employed   It is to be noted that Clark and Hall (1937) and Stewart (1938) have found *C bifermentans* antiserum of protective value against the pathogenic variety of this species (*Clostridium sordellii*)

No strains could be identified culturally or by pathogenicity tests as *Clostridium histolyticum*   In soil, Zeissler and Rassfeld (1928) reported an incidence of 2 per cent for this species   Smith and George (1946) working in Italy did not find strains of *C histolyticum*   In their series, Weinberg and Seguin (1918) isolated 8 strains from cases late in the investigation   In MacLennan's series (1943), all 9 patients with *C histolyticum* in the wound flora succumbed

Death from gas gangrene has been attributed generally to toxemia (MacLennan, 1946)   Bacteremia which is known to occur has been considered a terminal event, although this conclusion, drawn from Weinberg and Seguin's paper (1918), may not be warranted   Further studies on blood cultures in gas gangrene are needed to determine whether bacteremia is an additional factor in the high mortality rate which still exists in spite of present therapeutic agents and surgical technique   There is experimental evidence that is suggestive, for once clostridia had entered the blood stream in infected mice, which occurred after 3 hours, McIntosh and Selbie (1943a, 1943b) found local chemotherapy to be less effective

In 7 local anaerobic infections cultured in Italy, 7 strains of *C perfringens* and 5 of *C novyi* were found   Thus, pathogenic species of clostridia were commonly found and were not less frequent than proteolytic nonpathogenic clostridia   This is the opposite of the findings in a small series of cases of "anaerobic

cellulitis" cultured by MacLennan (1943) in the Western Desert, but agrees with those of Weinberg and Seguin (1918) in the cases called "gaseous phlegmon" or "gaseous wounds." In our experience, "heavy local anaerobic infection" used by Robertson (1929) may be a more accurate description of the lesions seen in Italy than "anaerobic cellulitis." Beginning with local anaerobic infections in dead tissue, all gradations and degrees of infection resulted, with fulminating gas gangrene at the extreme. Debridement removed infected tissue and often prevented further spread. Prophylactic penicillin was used in wounded patients in the latter part of the Italian campaign, but no data are available on its effect on the cultural findings of the bacterial flora of the wounds or on its therapeutic value in the dosage used.

Numerous nonpathogenic species of clostridia and many aerobes (not listed in our tables), particularly nonhemolytic streptococci, were found in the specimens from gas gangrene and local anaerobic infections but were not investigated further. No information was obtained on their significance.

SUMMARY

In 30 cases of gas gangrene cultured in Italy, 80 per cent of the cases showed *Clostridium perfringens*, 50 per cent *Clostridium novyi*, and 1 case *Clostridium septicum*. The high incidence of *Clostridium novyi* confirms the earlier reports of French and British investigators. *Clostridium tetani* and nonpathogenic *Clostridium bifermentans* were found. No strains of *Clostridium histolyticum* were identified. In a trial series, clostridia were recovered post mortem from blood cultures in 2 cases of gas gangrene. No data were obtained on the prognostic significance of a positive blood culture. Seven local anaerobic infections showed on culture 7 *Clostridium perfringens* and 5 *Clostridium novyi*.

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ROUGH-SMOOTH DISSOCIATION OF NEISSERIA INTRACELLULARIS

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Dissociation in the *Neisseria* group is infrequently reported, therefore the following occurrence may be worthy of note The culture was a stock strain of *Neisseria intracellularis* Gordon type III which had been maintained in stock by the author for about 2 years prior to the appearance of the variant   It had been cultivated for about a year on human blood agar slants and subsequently on Dorset's egg medium with frequent platings on blood agar

The variation was first noted on a plate of human blood agar containing 0 5 per cent glucose after 24 hours' incubation at 37 C and 24 hours at about 29 C The variation appeared to be of the simple R-S type   About 50 per cent of the colonies were typical meningococcus colonies and about 50 per cent were of the variant type   The R or variant colonies were about half the size of most of the typical S colonies   The surface of the R colonies was warty and the margins were irregular   These colonies had a heaped-up appearance and were pinkish or yellowish pink in color, in marked contrast to the typical or smooth colony When the R colony was picked off the medium, the entire structure came away intact   It was found to be extremely hard and could only be broken up by being ground between two glass slides

Transfers of the two colony types to blood agar plates of the same composition gave the following results   The typical S type colonies gave rise to pure cultures of S colonies through successive subcultures   The R or variant type gave cultures which consisted of about half typical colonies and half R colonies for five successive transplants   The smooth colonies from these plates invariably gave rise to pure cultures of typical colonies

Transfers to plates of 10 per cent ascitic fluid agar containing 0 5 per cent glucose resulted in 100 per cent typical smooth *Neisseria intracellularis* colonies with either type as an inoculum   Transfers to Avery's blood broth with subsequent streaking on the ascitic fluid agar also resulted in pure cultures of the S type of colony   Laked blood agar prepared from the same blood used for the blood agar plates resulted in half R and half S colonies, provided they were inoculated with material from an R colony

A sudden change occurred in transplants from the plates of the fifth successive passage of the R type of colony   The sixth successive passage on blood agar plates and laked blood agar plates gave a pure culture of a colony which resembled the original R, except in size   This new colony was much smaller than the original variant   At 24 hours it was microscopic in size, and at maximum develop-

ment (3 days) was about 1 mm in diameter   When these small R colonies were transferred to blood agar plates, they gave rise to pure cultures of similar colonies When transferred to ascitic agar plates they maintained their characteristics Growth in Avery's blood broth which was streaked on ascitic agar also gave pure culture of the small R type   This small R type of colony was carried for 10 successive generations on ascitic agar, blood agar, and Avery's blood broth without any indication of further change in colony morphology or any tendency to revert to the normal type of colony
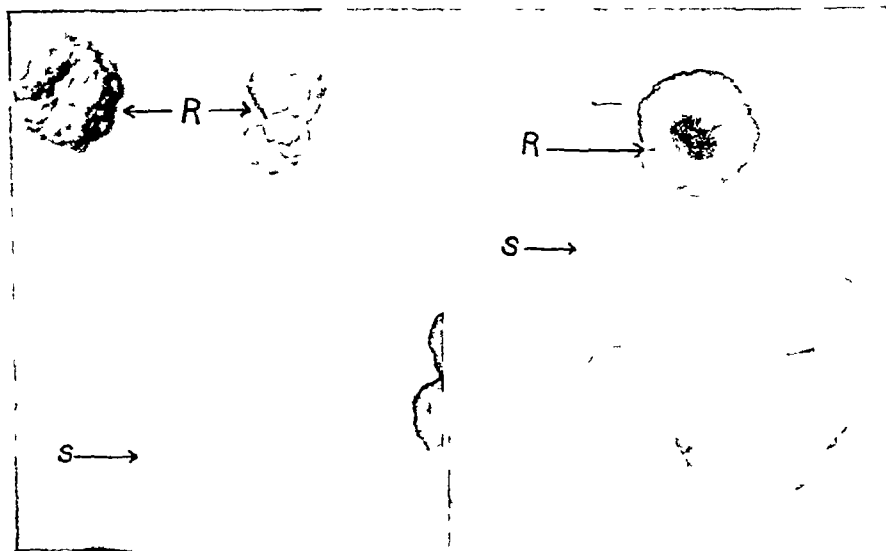
                    FIG 1   FIG 2
FIG 1 ROUGH SMOOTH DISSOCIATION   BLOOD AGAR PLATE, REFLECTED LIGHT
              MAGNIFICATION, APPROXIMATELY 10 DIAMETERS
FIG 2 ROUGH SMOOTH DISSOCIATION   BLOOD AGAR PLATE TRANSMITTED LIGHT
              MAGNIFICATION APPROXIMATELY 10 DIAMETERS

Microscopic examination of the various types of colonies showed them to consist entirely of gram-negative cocci and diplococci morphologically resembling Neisseria   Fermentation studies on the large R type could not be run because the cultures invariably reverted to the S type in the fermentation tubes   Such tests however showed the production of acid from glucose and maltose   The small R type which was stable, produced a faint acidity in glucose, but none in maltose sucrose lactose, dextrin, inulin, or xylose

The production of this variant was apparently due to the accidental use of human blood which contained antimeningococcus antibodies   This is substantiated by the following observations   First, the S or typical colony produced wide dense halos sometimes one-half to three-fourths inches in diameter on plates prepared with this specimen of blood   Secondly, 2 months after the original observation material from the same tube that had produced the dissociated colonies was plated on media of identical composition, except that the blood w is

from a different donor   It yielded a pure culture of typical S type *Neisseria intracellularis* and no R colonies could be found on numerous plates   Thirdly, plasma obtained by centrifuging some of the same blood that produced the dissociation gave the following results in the agglutination tests   When the antigen was the Gordon type III strain, grown on ascitic agar, agglutination was positive in a dilution of 1 320   A satisfactory suspension of the small R colonies could not be prepared, and therefore the test was not satisfactory with this antigen   Similar antigens of other strains of *Neisseria intracellularis* all gave negative agglutination with this plasma   These strains included the other Gordon types and several strains isolated locally from cases of meningitis

SUMMARY

A stable R variant of Gordon type III *Neisseria intracellularis* that has lost the ability to ferment maltose and that was apparently induced by antibodies is reported

DESCRIPTION OF STRAIN C27 A MOTILE ORGANISM WITH THE MAJOR ANTIGEN OF SHIGELLA SONNEI PHASE I

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The development of typing sera for antigenic analysis of *Salmonella* organisms has made possible the discovery of *Salmonella* antigens in cultures of other genera Reports of such antigenic interrelationships have become common in the literature Similarly, the development of typing sera for the detection of type-specific antigens in *Shigella* is making possible the discovery of antigenic relationships between *Shigella* and organisms of other genera

In a paper devoted to the typing of *Shigella*, Ferguson *et al* (1947) referred to a motile organism of the family *Enterobacteriaceae* which bears the major antigen of *Shigella sonnei* phase I, as defined by Wheeler and Mickle (1945) A detailed description of this organism, which has been given the strain designation C27, is contained in the present report

Isolation of the culture was effected on MacConkey agar plates from a fecal specimen submitted to this laboratory for culture No clinical history was indicated on the specimen blank, and efforts to obtain a history from the physician have been unsuccessful

The identification of culture C27 was attempted by the methods used routinely in our diagnostic laboratories Lactose-nonfermenting colonies were transferred from a MacConkey plate to triple sugar agar slants on which growth produced a *Shigella*-like reaction Upon test with highly absorbed *Shigella* sera representative of the types commonly encountered in Michigan, the growth from triple sugar slants reacted strongly in *Shigella sonnei* phase I antiserum No reaction occurred in any of the other sera Biochemical and other tests revealed, however, that C27 differed in several respects from *Shigella sonnei*

Strain C27 was found to be a gram-negative, nonsporulating rod form, actively motile when grown at room or incubator temperatures Motility was sufficiently pronounced that growth spread through a 6-inch column of semisolid agar in 12 hours Flagellar stain preparations, made after the method of Leifson (1938) with Baltimore Biological Laboratory stain, revealed that C27 was apparently amphitrichous

The organism grew readily at 37 C on nutrient agar, on SS, and MacConkey media, and on veal infusion blood agar On the latter medium, small but clear zones of hemolysis were produced around the massed growth or isolated colonies On blood agar the colonies appeared gray, shiny, and opaque, with slightly raised centers, smooth surfaces, and entire edges Colony size varied from 2 to 4 mm

Strain C27 was an anaerogenic culture which fermented glucose and maltose in 24 hours and produced acid from salicin in 7 days Sucrose, mannitol,

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sorbitol, dulcitol, rhamnose, and xylose were not attacked   Acetylmethylcar-
binol was not formed, and urea and citrate were not utilized   Indole and H₂S
were produced and trimethylamine oxide was reduced   The time of lactose
fermentation was variable   On first isolation, one subculture fermented lactose
after 72 hours, whereas a variant fermented the carbohydrate in 48 hours   The
latter, after serial transfer, fermented lactose in 24 hours

Immune sera for strain C27 were developed in two rabbits whose sera prior
to immunization contained no perceptible agglutinins for C27 or *Shigella sonnei*
phase I organisms   *Shigella sonnei* phase I antiserum unabsorbed and *S sonnei*
absorbed serum freed of cross reactions as described by Ferguson, Branston,
MacCallum, and Carlson (1947) were available for agglutination studies   The

TABLE 1

Serological results

	SERUM					
ORGANISM	*S sonnei*, phase I			C27		
	Unab-sorbed	Absorbed by		Unab-sorbed	Absorbed by	
		Shigella sp *	C27		*S sonnei*	C27 boiled
S sonnei, phase I living	10,240	10,240	<80	20,480	80	<80
S sonnei, phase I alcohol treated	2,560	—	—	5,120	<80	—
C27, living	2,560	2,560	<80	20,480	5,120†	1,280†
C27, alcohol-treated	2,560	—	—	5,120	<80	—

Agglutinations conducted at 50 C with overnight incubation, lowest dilution 1 80,—not
made

 * *Shigella* cross reactions removed

 † Soft, floccular clumps which shake out readily

results of such studies with absorbed and unabsorbed sera and the respective
antigens are recorded in table 1

It is obvious from a study of the table that C27 strain has a somatic antigen
similar to the major antigen of *Shigella sonnei* phase I   This is borne out by
the fact that C27 organisms were able to exhaust the specific agglutinins from
an absorbed *Shigella sonnei* typing serum   This is also borne out by further
data in the table which show that *Shigella sonnei* phase I organisms are capable
of exhausting the somatic agglutinins from C27 antiserum

Organism C27 possesses additional antigens not shared by *Shigella sonnei*
which are probably contained in the flagella   It will be seen in the table that
absorption of C27 antiserum with *Shigella sonnei* organisms removed agglutinins
for *S sonnei*, while a considerable residual antibody content remained for C27
untreated culture   The clumps formed by agglutination of both agar- and
broth-grown suspensions of C27 with the residual agglutinins were soft and
floccular—very much like the clumps formed by agglutination of *Salmonella*
organisms with pure "H" antisera   Moreover, a suspension of C27 after treat-

ment with absolute alcohol after the method of Edwards and Bruner (1942) would not react with this same absorbed serum   Further evidence that a labile antigen not shared by *Shigella sonnei* is present in the C27 culture was demonstrated by absorption of C27 serum by a boiled suspension of the homologous organism Although agglutinins for *Shigella sonnei* were removed by this treatment, residual agglutinins were left for the C27 culture

Our conclusion that the C27 strain is related to *Shigella sonnei* phase I rather than phase II is based on examination of C27 organisms with phase I and phase II sera furnished by Dr K M Wheeler, and on the outcome of tests with sera produced by us   A culture of C27 examined by Dr Wheeler was found to react with his phase I absorbed serum   The culture of *Shigella sonnei* used by us for the production of sera and for this study is remarkable for its stability in phase I   It was carefully checked for phase throughout the study

Since the finding of the C27 culture, a second motile, paracolonlike organism related to *Shigella sonnei* phase I has been discovered by Wheeler (personal communication)   It appears possible that similar cultures may be found as the use of absorbed *Shigella* typing serum becomes widespread

SUMMARY

A motile organism of the family *Enterobacteriaceae* containing a somatic antigen similar to the major antigen of *Shigella sonnei* phase I is described   Biochemically this organism appears to be an anaerogenic paracolon

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MORPHOLOGY OF ESCHERICHIA COLI EXPOSED TO PENICILLIN AS OBSERVED WITH THE ELECTRON MICROSCOPE

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The morphological effects of penicillin on gram-negative nonsporeforming rods, especially *Escherichia coli*, have been studied by several authors   Gardner (1940) reported that grotesque forms of *E coli* resulted from autolytic swelling when this organism was treated with penicillin and that elongated, swollen cells resulted from incomplete fission   Weiss (1943) showed by electron micrographs that certain bacterial cells, when medicated with penicillin, became enlarged and fission was often incomplete   The effects of penicillin on intestinal bacteria as reported by Thomas and Levine (1945) included long twisting filaments in lower inhibitory concentrations and cells resembling Pasteur flasks, swelled fusiform rods, large globular cells, and irregular masses in higher concentrations   In concentrations just above those with visible growth, globular masses were found upon centrifugation and examination of the sediment   Normal rods were cultivated from the masses in the sediment   In the same year Alture-Werber, Lipschitz, Kashdan, and Rosenblatt (1945) studied the effect of incompletely inhibitory concentrations of penicillin on *Escherichia coli*   These authors found organisms resembling budding fungi in the urine of patients treated with penicillin   Culturing for molds was negative, but *E coli* was isolated on other media and it was concluded that penicillin was responsible for the funguslike appearance of the cells in urine   This assumption was confirmed by *in vitro* experiments using MacConkey's agar to which was added varying concentrations of penicillin   Morphological changes noted were diphtheroidlike, bipolar cells at 75 units per ml , unsegmented filaments with mycelialllike appearance at 100 units per ml, and at 150 units per ml forms similar to those observed in the urine specimens, designated as zygosporelike bodies   Kojima and Heimbrock (1946) and Fennel (1946) confirmed the findings of Alture-Werber *et al* (1945)   Both reports indicated that the urine of penicillin-treated patients contained budding funguslike forms, which in one case were identified as *B aerogenes* (*Aerobacter aerogenes*) and in the other as *E coli*   Kojima and Heimbrock did not obtain bulbous forms in broth cultures with penicillin, but Fennel found various bizarre types in glucose broth containing various concentrations of penicillin   In all of the foregoing cases, when the organisms showing atypical forms were cultured on media not containing penicillin, only normal rods were found   A short but informative review of the action of penicillin and its effect on bacterial morphology was given by Fisher (1946)

Morphological variation may be induced by agents or conditions other than by the use of penicillin   Only a few of the more important aspects of the phe-

nomena of variation need be mentioned here   Scales (1921) found several morphological types of *E coli* including coccoidal types resembling those re ported in this paper, but induced by 6 per cent sodium chloride   The influence of H ion concentration on the structure of *Hemophilus influenzae* was noted by Reed and Orr (1923)   Long filaments, coccuslike forms, and a variety of swollen and elongated cells were found at pH 6 5 to the maximum acid pH allowing growth, and from pH 8 0 to the maximum alkaline pH allowing growth   In his series of studies on microbic heredity, Mellon (1925a, 1925b, 1926), observed a funguslike organism in the urine of patients treated with utropin and sodium acid phosphate   The organism was found to be *E coli* on ordinary media, but "zygospore" formation was noted on media with inducing substances added Coccoid forms followed by coarse filaments and rods arose from the "zygospores " The pleomorphism of *B paratyphi* B (*Salmonella schottmuelleri*) as reported by Kritschewski and Ponomarewa (1934) is especially noteworthy because they apparently used no inducing substances, having cultured the organisms on 1 to 2 per cent raffinose agar   Their photographs give excellent evidence of the variations of form of bacterial cells   Wahlin and Almaden (1939) covered the so-called "megalmorphic phase" of bacteria in detail which the interested reader may find informative   No attempt is made here to review all of the works of Dienes and Klieneberger, but it is evident from their work that bacterial varia tion under normal conditions can be demonstrated by proper techniques   The appearance of fusiform bodies in colon bacillus colonies (Dienes, 1939a), in L organisms of Klieneberger and *Streptobacillus moniliformis* (Dienes, 1939b), in a *Flavobacterium* (Dienes, 1942), in a parainfluenza bacillus (Dienes, 1944), and in *Proteus* cultures (Dienes, 1946) is of interest due to the resemblance between these forms and those induced by penicillin in the organisms reported here and by others   The association of pleuropneumonialike organisms with *Strepto- bacillus moniliformis* as reported by Klieneberger (1942) and the pleomorphism of *Bacteroides* strains as shown by Dienes and Smith (1944) are also pertinent

MATERIALS AND METHODS

The methods used to demonstrate the effects of subinhibitory concentrations on *E coli* were virtually the same as those of Alture-Werber *et al* (1945)   Mac- Conkey's agar (Difco) was sterilized in 10-ml amounts in tubes and just before the plates were poured appropriate amounts of penicillin[1] were added to give final concentrations ranging from 50 to 200 units per ml   *E coli* strain 252, University of Illinois stock culture collection, was employed as the test strain, 0 1 ml of a 1 100 dilution of a 24-hour-old culture being added to each tube The plates were incubated at 37 C for 18 to 24 hours and gram stains made from isolated colonies obtained at the various concentrations of penicillin   Suitable colonies were selected and a suspension was made for use in preparing mounts for the electron microscope   The mounts were prepared in the usual manner using a collodion membrane, and examination of the specimens was made with the type B, RCA electron microscope   In order to demonstrate similar

[1] Na Penicillin used was supplied by the Schenley Laboratories, Lawrenceburg, Indiana

ties and differences between light and electron microscopy of the same type of cells, strain 252 was grown on MacConkey's agar as follows. About 0 1 ml of melted MacConkey's agar containing a suitable concentration of penicillin was pipetted onto a sterile glass slide and immediately covered with a sterile cover slip. The latter was sealed with melted paraffin and the slide culture then incubated either on a warm stage at 37 C or placed in sterile petri plates at 37 C. Frequent examinations were made at intervals until the desired forms could be observed and photographed under oil immersion using a Leitz-Wetzler "makam" attached to a Leitz-Wetzler microscope, Wratten M plates were used as negatives.

RESULTS

E coli, strain 252, was found to grow abundantly on MacConkey's agar plates in concentrations up to 100 units per ml and to a lesser degree in concentrations up to 200 units. In the lower concentrations there was little change in the morphology, but as the unit age increased more elongated and swollen cells appeared, many of them remaining only partially divided. The appearance of very large fusiform bodies was especially noted at 150 and 200 units per ml on the agar plates. They were also readily found in the slide cultures at 50 units per ml, but at this concentration there was also an abundance of normal rods, whereas with the higher concentrations nearly all of the cells were elongated and had fusiform swellings.

Several preparations were made from the agar plates and examined with the electron microscope. It was soon found that low magnification (3,000 ×) gave the best results because the fusiform bodies and elongated cells were so large. The photographs shown were made from a specimen taken from Mac-Conkey's agar plate containing 150 units per ml after incubation at 37 C for 18 hours.

Figure 1 (no A) shows the appearance of a young fusiform body near the lower right-hand corner. The entire cell is dense and apparently homogeneous, whereas the older cells as shown toward the upper right-hand corner are entirely granulated. Elongated and partially divided dense cells may also be seen. The granular appearance surrounding the cells may or may not be of significance and this has not been determined at this time. Figure 1 (no B) shows another type of cell commonly encountered and reveals the intense granulation which can be observed in less degree with the light microscope. Numbers C and D (figure 1) represent a type of granule found throughout the entire specimen, but again the significance of these bodies is not yet clear. Forms similar to these may be found in the slide cultures not only of strain 252 but of several others studied. Figure 1 (no E) shows two cells only somewhat larger than normal cells, but the failure of the cells to separate is clearly seen plus the granulation common to older cells. Figure 2 (no A) again shows partially divided cells, probably of normal size, and close examination reveals the presence of numerous flagella. It might be pointed out that this strain of *E coli* is actively motile and that the elongated and fusiform cells are apparently likewise motile. Figure 2 (no B)

Fig 1

EXPLANATION OF FIGURES

The photographs in figures 1 and 2 and no A figure 3 were all taken at a magnification of 3,000 × on the electron microscope and enlarged 4 × photographically Number B figure 3 was taken of living cells under oil immersion (97 ×) with a 10 × ocular and presented as a contact print, magnification approximately 970 ×
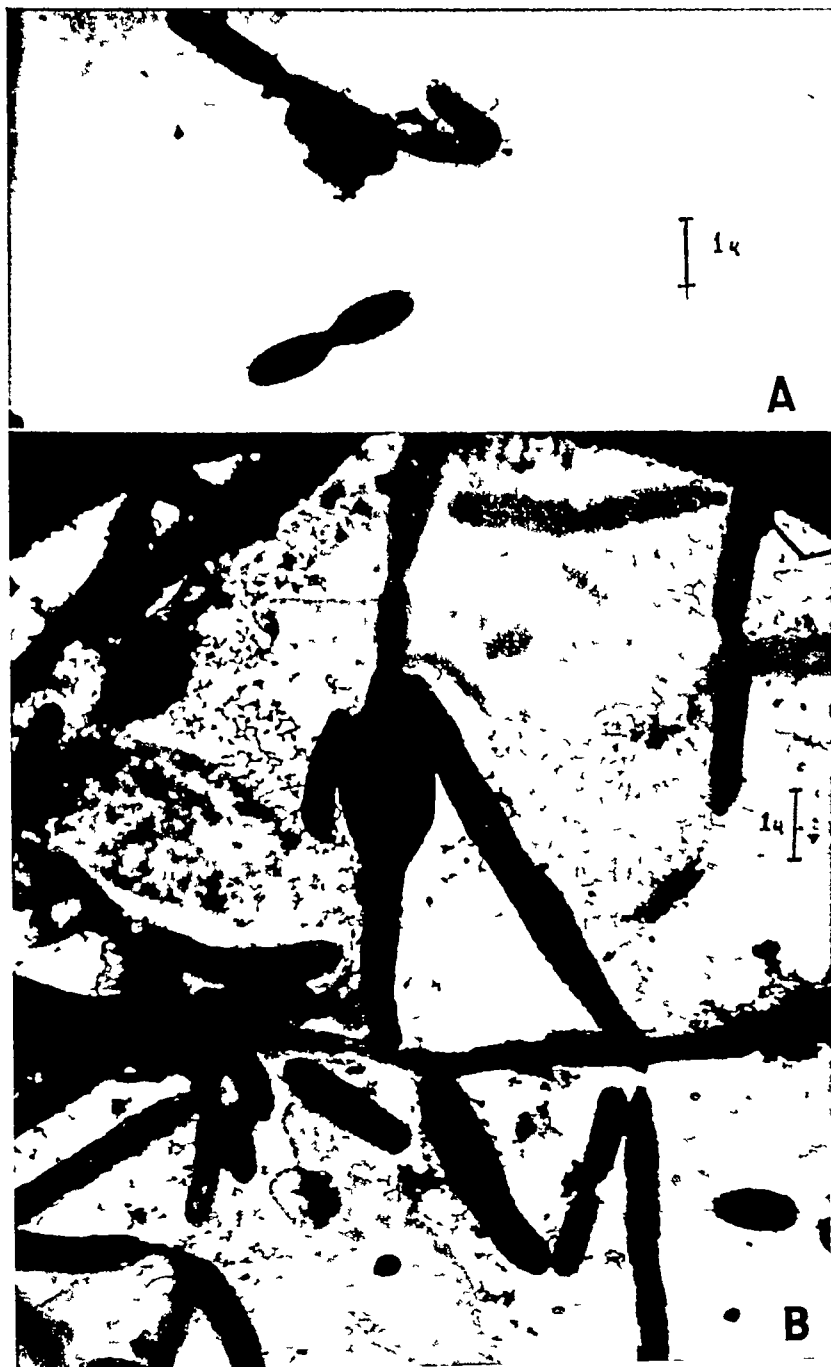
FIG 2

lepresents a typical field encounteied with this specimen   The presence of arti-
facts and extraneous mateiial could not be avoided   Figuie 3 (no A) repre-

sents another type of form frequently found, being considerably elongated and filamentous with one or several swellings along the cell although only one swollen portion is shown Figure 3 (no B) is included to show the appearance of this same strain of *E coli* when grown on MacConkey's agar slide cultures with 50
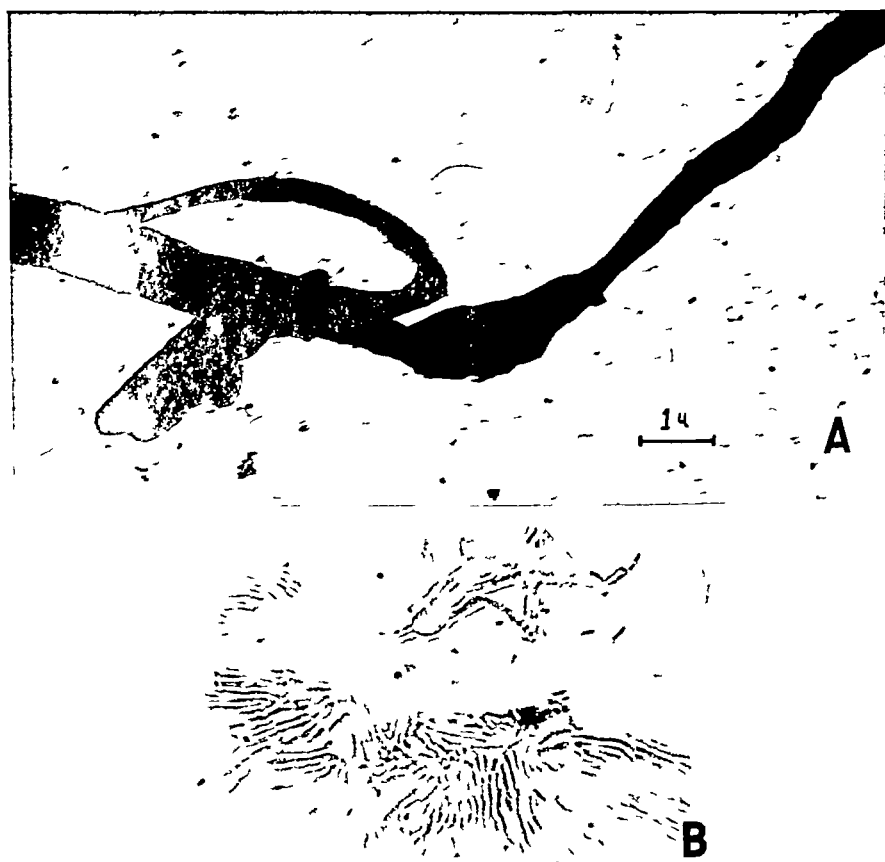
FIG 3

units per ml of penicillin The intense granulation of the 18-hour-old cells is quite evident although not so well revealed as by the higher magnification obtained with the electron microscope

DISCUSSION AND SUMMARY

Several electron microscope pictures are presented, as well as one light microscope photograph revealing the form and inner structure of some of the types of cells induced by exposure to penicillin, using a strain of *Escherichia coli* Observations under oil immersion with light microscopy indicate that the fusiform bodies arise by direct swelling of a portion of an elongated rod No attempt is made at the present time to interpret the significance of the intense

granulation of the fusiform and rod forms as revealed by electron microscopy nor to account for the fate of these cells Work is being continued along these lines and will be reported at a later date

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ACETIC ACID PRODUCTION FROM ETHANOL BY FLUORESCENT PSEUDOMONADS

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Apart from the observation by Alsberg (1911) that gluconic acid is produced from glucose by *Phytomonas savastanoi*, the oxidative metabolism of fluorescent pseudomonads has received little attention until very recently   For the most part it has been tacitly assumed that these organisms, by virtue of their obligately aerobic nature, carry out a complete mineralization of organic substrates (den Dooren de Jong, 1926)   However, the researches of Pervozvanski (1939a, 1939b), followed by those of Lockwood et al   (1941, 1946), have established the unexpected fact that the dissimilation of monosaccharides by the majority of fluorescent pseudomonads is accompanied by the production and accumulation of the corresponding hexonic or pentonic acids in large amounts   Some strains when acting on glucose also carry the oxidation further to 2-ketogluconic acid (Pervozvanski 1939a, Lockwood et al   1941) and α-ketoglutaric acid (Lockwood and Stodola, 1946)   The failure of all previous investigators except Alsberg to observe these phenomena may be ascribed to the use of weakly buffered and insufficiently aerated media (e g , the customary tubes of carbohydrate broth), since acidity and poor oxygen supply are both limiting factors for the transformations in question

   The oxidation of monosaccharides to the corresponding -onic acids is a pattern of biochemical behavior that occurs elsewhere among bacteria, so far as is at present known, only in the *Acetobacter* group   Coupled with the frequently overlooked morphological similarities, it serves, as Vaughn (1942) has pointed out, to indicate a close relationship between the genera *Acetobacter* and *Pseudomonas*   Consequently it seemed of interest to find out whether the primary biochemical characteristic of the acetic acid bacteria, namely the oxidation of ethanol to acetic acid, might not also exist in the genus *Pseudomonas*

MATERIALS AND METHODS

   Thirteen strains of fluorescent pseudomonads were studied, of which one was a strain of *Pseudomonas aeruginosa* and the remainder belonged to the *Pseudomonas fluorescens* species-group [1]   Three cultures (designated by the prefix NRRL) were received from Dr Lockwood, by whom they had been used in studies on the metabolism of monosaccharides   The others (designated by the prefix A 3) were isolated locally from soil, using the customary enrichment methods (den Dooren de Jong, 1926)

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[1] The term "*P fluorescens* species-group" is used to designate pseudomonads producing a fluorescent pigment but devoid of accessory phenazine pigments (pyocyanin, chlororaphin, etc)   The taxonomic criteria in current use with this group are inadequate, in my opinion, to justify any further specific subdivisions

Ability to use ethanol as sole carbon source was tested by streaking on mineral agar plates (0 1 per cent NH_4Cl, 0 1 per cent K_2HPO_4, 0 05 per cent $MgSO_4$, and 1 5 per cent agar) containing 1 0 per cent ethanol, and comparing growth with that on a control plate devoid of carbon source Preliminary observations on acid production from ethanol were made by streaking on mineral or peptone agar plates containing ethanol and $CaCO_3$ and noting the formation of cleared zones in the carbonate around the bacterial growth This method is also extremely useful for a rough screening of strains that produce acid from sugars

For quantitative studies on ethanol oxidation, the organisms were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium Incubation was at 30 C on a shaking machine The medium consisted of 0 5 per cent Difco peptone with various concentrations of ethanol and, in some experiments, also 0 5 per cent $CaCO_3$

TABLE 1

Acetic acid production from ethanol by strains of the
P fluorescens species-group after 5 days

STRAIN	RESIDUAL ETHANOL	ETHANOL USED	ACETIC ACID FORMED	YIELD OF ACETIC ACID[a]
	mg	*mg*	*mg*	
Uninoculated	536			
A 3 1	82	454	58	10
A 3 2	0	536	242	35
A 3 3	21	515	145	21
A 3 6	0	536	313	45
A 3 8	152	384	365	71
A 3 9	0	536	45	6
A 3 10	48	488	71	11
NRRL B-13	24	512	5	1

Medium 0 5 per cent peptone, 0 5 per cent $CaCO_3$, and 1 0 per cent ethanol
[a] Expressed as percentages based on ethanol oxidized

Ethanol was determined by dichromate oxidation of neutral distillates and estimation of residual dichromate, acetic acid, by titration of steam distillates The acetic acid was identified by the iodine-lanthanum reaction and by formation of the characteristic copper salt (Meyer, 1933, p 101)

RESULTS

Nine of the 13 strains were capable of developing abundantly on mineral, ethanol agar with ethanol as the sole carbon source The remaining 4 (including two—NRRL B-14 and B-25—received from Dr Lockwood) failed to develop on this medium Of the 9 positive strains, 7 produced sufficient acid on mineral, ethanol, $CaCO_3$ agar to cause a marked dissolution of the carbonate, and one more (NRRL B-13) produced a very slight amount of acid The only ethanol-utilizing strain which failed to produce any acid whatsoever was the isolate of *P aeruginosa* Ethanol also gave rise to acid production when the mineral base

was replaced by 0 5 per cent peptone, indeed, under these conditions slightly more acid appeared to be formed

Quantitative data on ethanol oxidation and acetic acid formation by the 8 acid-producing strains are shown in table 1   The medium contained 0 5 per cent CaCO₃ and slightly over 1 per cent ethanol   It can be seen that the degree of acetification varies very greatly from strain to strain   Some carry out a virtually complete oxidation of the ethanol with negligible accumulation of

TABLE 2

Total titratable acidity and final pH produced by three strains of
fluorescent pseudomonads when grown in 50 ml of peptone, 1 5 per cent ethanol broth

STRAIN	TITRATABLE ACIDITY ML OF 0 1 N			FINAL pH
	36 hr	60 hr	84 hr	84 hr
Uninoculated	2 0	2 0	2 0	7 35
A 3 1	1 5	8 5	8 5	4 55
A.3 2	1 3	10 1	10 1	4 50
A 3 8	7 0	7 0	7 0	4 95

Cultures grown at 30 C on a shaking machine

TABLE 3

Acetic acid production from ethanol by strain A 3 8

MEDIUM*	FINAL pH	TITRATABLE ACIDITY ML of 0 1 N	RESIDUAL ETHANOL	ETHANOL USED	ACETIC ACID FORMED
			mg	mg	mg
Peptone, control	7 15	1 6			
Peptone, inoculated	8 80	0 0			
Peptone, ethanol, control	7 15	1 8	535		
Peptone, ethanol, inoculated	4 55	10 0	276	259	50
Peptone, ethanol, CaCO₂, control	7 75		555		
Peptone, ethanol, CaCO₂, inoculated	5 00		57	498	231

Cultures were grown for 5 days at 30 C on a shaking machine
* Constituents of the medium were used in the following concentrations   peptone, 0 5 per cent, ethanol, 1 0 per cent, and CaCO₃, 0 5 per cent

acetic acid, whereas others convert a substantial proportion of the ethanol oxidized into acetic acid

In the absence of CaCO₃, acid production (as gauged by titratable acidity) is slight, even with the most actively acetifying strains   This is owing to the fact that the pH soon drops below 5 0 and the organisms die off   Typical figures for titratable acidities and final pH in peptone ethanol broth cultures are given in table 2   Streaked plates made from such cultures after 3 to 4 days reveal the presence of very few viable cells   A somewhat more detailed picture of the effect of CaCO₃ addition is given for strain A 3 8   in table 3

DISCUSSION

The present demonstration that some fluorescent pseudomonads can produce substantial amounts of acetic acid from ethanol might have been predicted in the light of recent work on their metabolism   Although acetification is not a universal property of these organisms, some strains being unable to attack primary alcohols at all, its very existence in the *P fluorescens* species-group raises a nice taxonomic problem, since the family *Acetobacteriaceae* and the genus *Acetobacter* are currently segregated from other pseudomonads primarily on the basis of their ability to produce acetic acid from ethanol   In view of the extensive morphological and biochemical parallelism between acetic acid bacteria and organisms of the *P fluorescens* type, it seems indefensible any longer to maintain a family *Acetobacteriaceae*, its members should be incorporated in the family *Pseudomonadaceae*   The genus *Acetobacter*, if it is to be kept at all, must be redefined in a manner which no longer stresses so exclusively the fact of acetification   As an additional differential property, acid tolerance, which is so marked in these organisms as contrasted with other heterotrophic pseudomonads, should be considered

SUMMARY

Certain strains of the *Pseudomonas fluorescens* species-group can oxidize ethanol with the production and accumulation of acetic acid   The intensity of acetification varies greatly from strain to strain   Acetification proceeds best in a medium well buffered with calcium carbonate   In poorly buffered media, ethanol oxidation is soon checked by increasing acidity

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GROWTH RESPONSES OF A SULFONAMIDE-REQUIRING MUTANT STRAIN OF NEUROSPORA[1]

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A mutant strain of *Neurospora crassa* has appeared in which the antagonistic roles of *p*-aminobenzoic acid and the sulfonamides have been reversed to a considerable extent   Optimal growth of this strain occurs only in the presence of sulfonamides   Conversely, *p*-aminobenzoic acid is a potent fungistatic agent for this strain under certain conditions

To say that sulfanilamide has become an essential metabolite and *p*-aminobenzoic acid an inhibiting analog would be to oversimplify the altered physiology of this mutant strain   It will be shown that, in this strain, both sulfonamides and *p*-aminobenzoic acid are essential for growth, and that each acts as an inhibiting analog of the other   These interrelations are further complicated by the effect of temperature on the need for sulfonamides, and on the inhibition by *p*-aminobenzoic acid

The present report deals exclusively with the growth responses of this mutant strain to sulfonamides, to temperature, and to *p*-aminobenzoic acid   At the present time nothing definite is known of the physiological role of sulfonamides in this strain

MATERIALS AND METHODS

Methods   The procedures followed are essentially those described in a previous report (Emerson and Cushing, 1946)   Growth responses are recorded as growth rates, which were determined by the tube method of Ryan, Beadle, and Tatum (1943)

Symbols   For the sake of clarity and brevity, the following symbols will be used

PABA—*p*-aminobenzoic acid, H_2N ⟨ ⟩ COOH

SA   —sulfanilamide, H_2N ⟨ ⟩ $SOONH_2$

pab   —"*p*-aminobenzoicless," a gene interrupting the synthesis of PABA, strain 1633 of Tatum and Beadle (1942)

+^{pab} —the wild-type allele of pab

sfo   —"sulfonamide-requiring," a gene carried by strain E-15172 described in this paper

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$+^{sfo}$ —the wild-type allele of sfo

S-T —"sulfanilamide tolerant," a gene for resistance to SAN, strain C-40
     (Emerson and Cushing, 1946)

$+^{S\,T}$ —the wild-type allele of S-T

Origin of sulfonamide-requiring strain   In a previous communication (Emer
son and Cushing, 1946) mention was made of a mutant strain (E-13190) which
apparently required sulfonamides for growth   Mutant E-13190 appeared as a
segregant in one ascus of a cross between the sulfanilamide-tolerant strain and a
wild-type strain [C-40(E-8577)A × E-5297a]
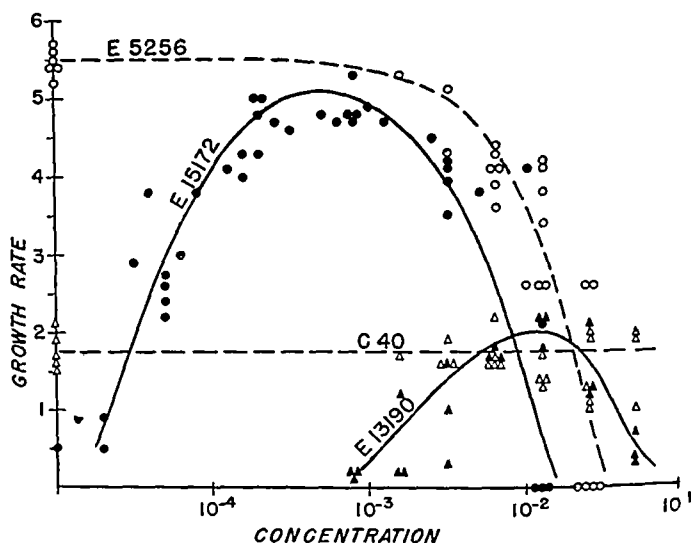
FIG 1 GROWTH RATES (IN MILLIMETERS PER HOUR) OF FOUR GENETICALLY
DIFFERENT STRAINS ON VARYING CONCENTRATIONS OF SULFANILAMIDE
AT 35 C
E-5256, wildtype ($+^{sfo}$ $+^{s-r}$), E-15172, sulfonamide-requiring strain (sfo $+^{s-r}$), C-40,
sulfanilamide-tolerant strain ($+^{sfo}$ S-T), E-13190, double mutant, sulfanilamide tolerant
and sulfonamide-requiring (sfo S-T)

Mutant strain E-13190 proved to be a "double mutant" carrying the gene for
sulfanilamide tolerance (S-T) characteristic of strain C-40 as well as the new
mutant gene (sfo) for sulfonamide requirement   In an outcross of strain
E-13190A to wild type (Abb-12a) these two genes segregated independently
The gene responsible for sulfonamide requirement was isolated from this cross as
E-15172A   The four different genetic constitutions resulting from this cross are
identified by their responses to varying concentrations of SA (figure 1)   The
double mutant (sfo S-T) has the maximal growth rate of about 2 mm per hour
characteristic of the S-T strain and requires about 50th molar SA for optimal
growth at 35 C   By itself sfo has a maximal growth rate of over 5 mm per hour,
similar to that of wild-type, and grows optimally on a much higher dilution of
SA

Genetic tests show that sfo lies very close to the centromere of a different chromosome from that carrying S-T   Both these genes are independent of pab, which is located some distance from the centromere of an undetermined chromosome

<center>RESULTS</center>

Substances stimulating growth in strain E-15172   The sulfonamide-requiring strain is able to utilize each of the sulfonamides that have been tested (figure 2) though the concentration necessary for optimal growth is different for different drugs   Growth was also supported by p-sulfamido-phenylalanine,[3] but never to maximal extent, perhaps because of inhibition resulting from competition between this analog and phenylalanine (cf   Mitchell and Niemann, 1947)
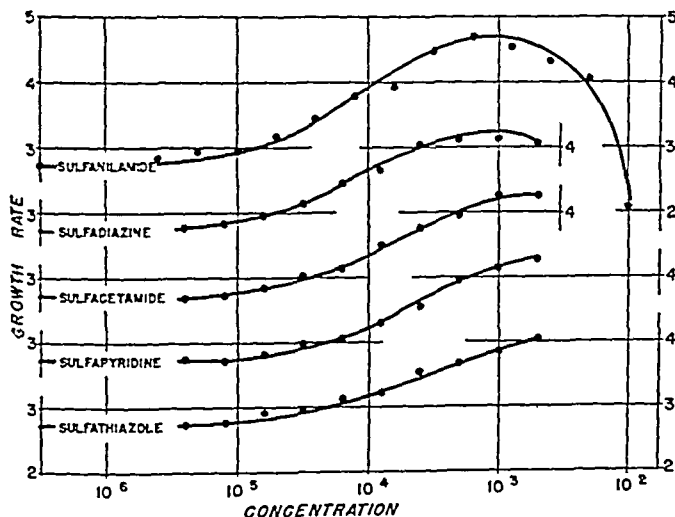
FIG 2 GROWTH RATES (IN MILLIMETERS PER HOUR) OF SULFONAMIDE-REQUIRING STRAIN SFO, ON VARYING MOLAR CONCENTRATIONS OF DIFFERENT SULFONAMIDES AT 30 C
See discussion on temperature effect

Methionine, the sulfone and sulfoxide of methionine, and taurine were unable to support growth of this strain, though methionine and its sulfoxide are utilized by certain other strains which require an organic source of sulfur (Horowitz, unpublished)

Effect of temperature on sulfonamide requirement   Although the sulfonamide-requiring strain will not grow at 35 C unless sulfonamides are present, considerable growth occurs at lower temperatures in the absence of sulfonamides   Data from experiments in which SA concentration and temperature were varied simultaneously are summarized in a contour graph in figure 3   In this diagram SA concentration increases from about millionth molar at the left to hundredth molar at the right   Temperatures increase from 25 C at the bottom of the diagram to over 36 C at the top   The contour lines pass through intersections of temperatures and concentrations at which equal growth rates occur

[3] The p-sulfamido phenylalanine was kindly supplied by Professor Carl G Niemann
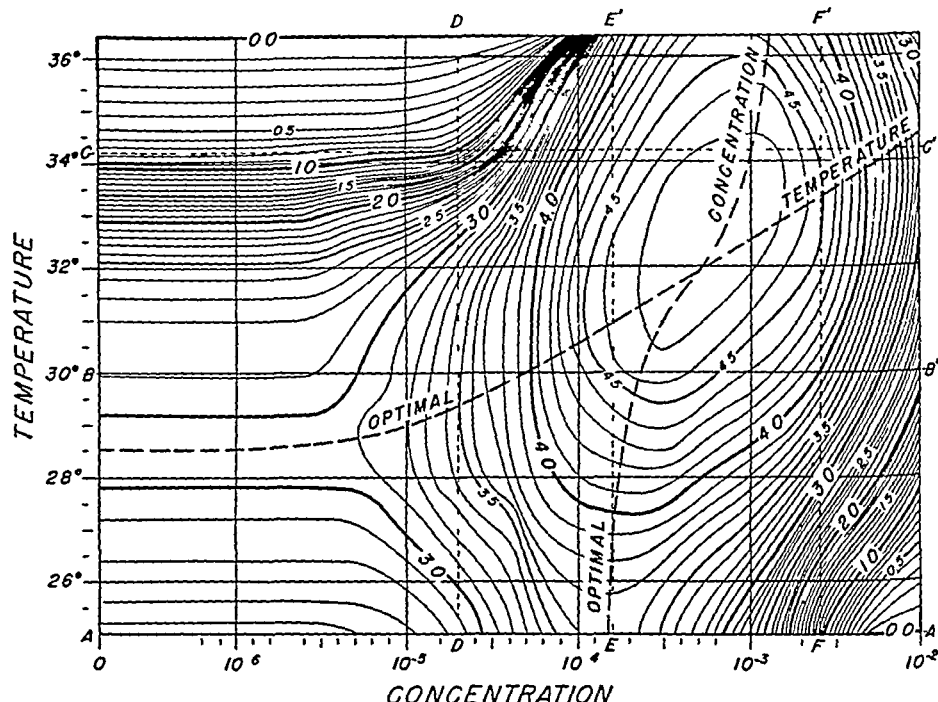
FIG 3 GROWTH RATES OF SULFONAMIDE-REQUIRING STRAIN, SFO, WITH VARYING
TEMPERATURE AND SA CONCENTRATION
Contour lines pass through points having equal growth rates (expressed as millimeters
per hour)   Concentrations are expressed as moles per liter   Rates were determined at
25, 27 8, 30, 32, 34 2, and 36 4 C, and at twofold dilutions from M/100 to M/1,638,400, for a
total of 96 different combinations of temperature and concentration, one quarter of which
were run in duplicate
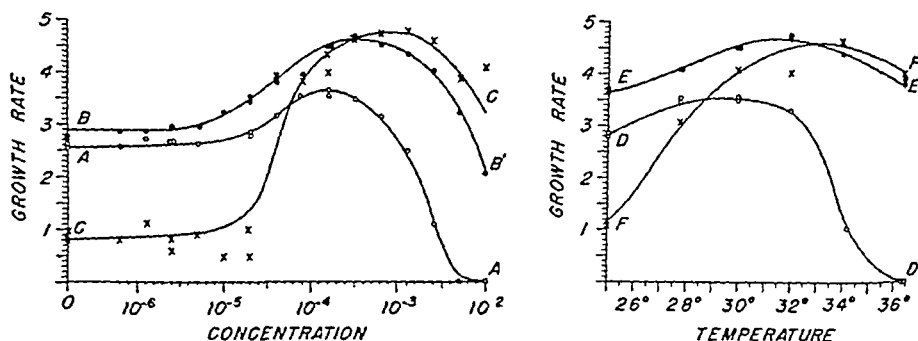
FIG 4 GROWTH RATES OF THE SULFONAMIDE-REQUIRING MUTANT, SFO, ON VARYING
CONCENTRATIONS OF SA (AT LEFT), AND AT VARYING TEMPERATURE (AT RIGHT)
Curve A-A', 25 C, B-B', 30 C, C-C', 34 2 C   Curve D-D', M/51,200, E-E', M/6,400, F-F',
M/400   The curves represent sections through the graph in figure 3 along the lines A-A',
B B', E-E', etc   The points represent observed values

Sections through this graph parallel to the base give curves showing the varia-
tions in growth rates with changing SA concentration at constant temperatures
Three such sections are reproduced in figure 4   Sections parallel to the sides of

the graph (figure 3) result in curves showing variations in growth rates with changing temperature at particular concentrations of SA   Three such sections are reproduced in figure 4

Growth rates are fairly constant from experiment to experiment throughout most of the range covered by the graph in figure 3   However, when growth is retarded by high concentration or by high temperature, the rates are much less constant and reproducible (note points on curve C-C' in figure 4)
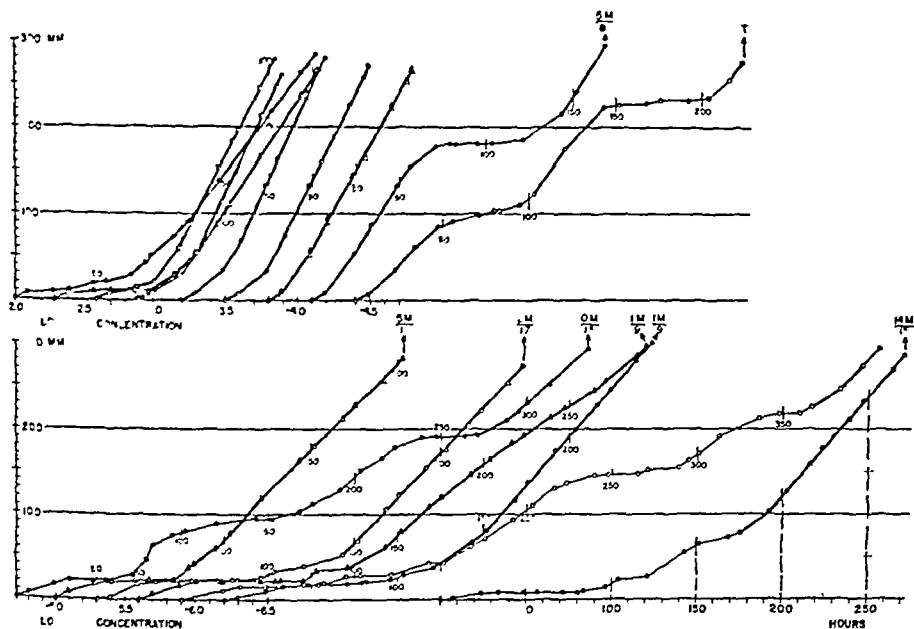
FIG 5 GROWTH CURVES OF THE SULFONAMIDE-REQUIRING STRAIN, SFO,
ON DIFFERENT CONCENTRATIONS OF SA AT 36 4 C
  The position at which a curve arises along the base line indicates the molar concentration of SA for that curve Over the remainder of the curve, horizontal distance represents elapsed time in hours, vertical distance represents total growth in millimeters Heavy lines indicate that growth was luxuriant, with well defined frontiers, lighter lines that growth was "feathery" The arrows indicate transfers to fresh growth tubes lacking SA T, transient, or nonpersistent reversion as shown by such transfers, the fractions show the number of mutant nuclei among the total nuclei tested in outcrosses following such transfers

Reversions   When the growth of the sulfonamide-requiring strain (sfo) is depressed by simultaneous high temperature and low SA concentration (cf figures 3 and 4) the additional complication of reversion is encountered   By "reversion" is meant a fairly abrupt change in growth rate and habit from those characteristic of the mutant strain to those closely resembling wild-type

Growth curves illustrating the character of the growth before and after reversion are reproduced in figure 5   At low SA concentrations (M/10,000 or less) and high temperatures (34 C or over), growth is characteristically light and "feathery," with no well-defined frontier   Such growth is represented by the lighter lines in figure 5   After reversion occurs, the growth of the fungus is
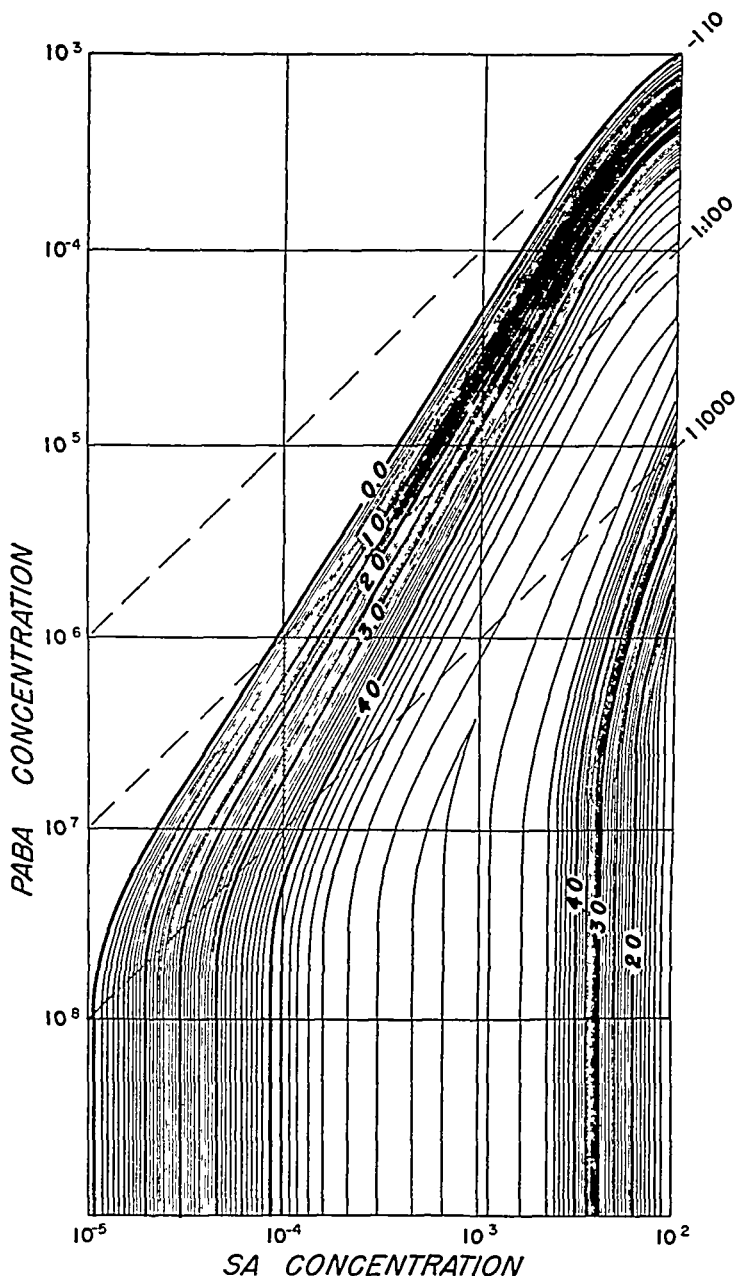
FIG 6 CONTOUR GRAPH SHOWING GROWTH RATES OF THE SULFONAMIDE-
REQUIRING STRAIN, SFO, AT 35 C ON VARYING MOLAR CONCENTRATIONS
OF SA AND PABA
Growth rates are indicated by the contour lines   The ratios in the upper right margin
are the molar ratios of PABA to SA   Based on determinations at 60 different combinations
of SA and PABA concentrations

luxuriant, and has a sharply defined frontier, such growth is represented by the
heavier lines in figure 5

Conidial transfers from the ends of growth tubes showing reverted growth (designated by arrows in figure 5) indicated that the reversions were persistant for the most part (see discussion of persistant "adaptive" changes in Emerson
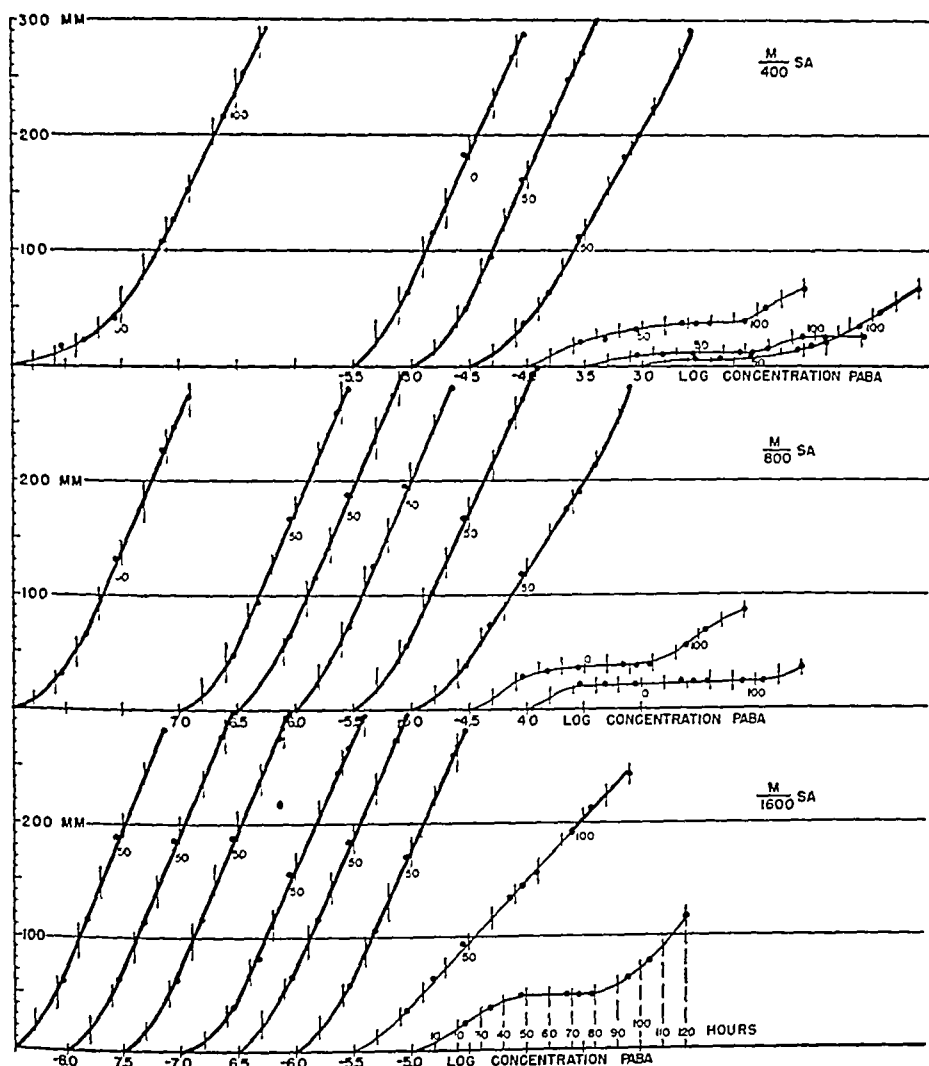
FIG 7 FAMILIES OF GROWTH CURVES OF SFO STRAIN ON VARYING
CONCENTRATIONS OF PABA

Upper set of curves in the presence of M/400 SA, middle set, M/800, lower set, M/1,600
Positions of the origins of the curves along the base line indicate the concentrations of
PABA for each   In each curve, horizontal distance represents elapsed time in hours,
vertical distance represents total growth in millimeters   Heavy lines represent mycelial
growth possessing definite frontiers, lighter lines represent "feathery" growth, with no
well defined frontier

and Cushing, 1946)   Transfers from such tubes to fresh tubes containing no
SA generally resulted in growth resembling that of wild-type without any pre-
liminary "feathery" stage such as is characteristic of sfo   Furthermore, crosses

from such reverted cultures generally showed that reversion had been accompa-
nied by mutation at a locus distinct from that responsible for sulfonamide re-
quirement   The fractions at the tops of growth curves in figure 5 show the num-
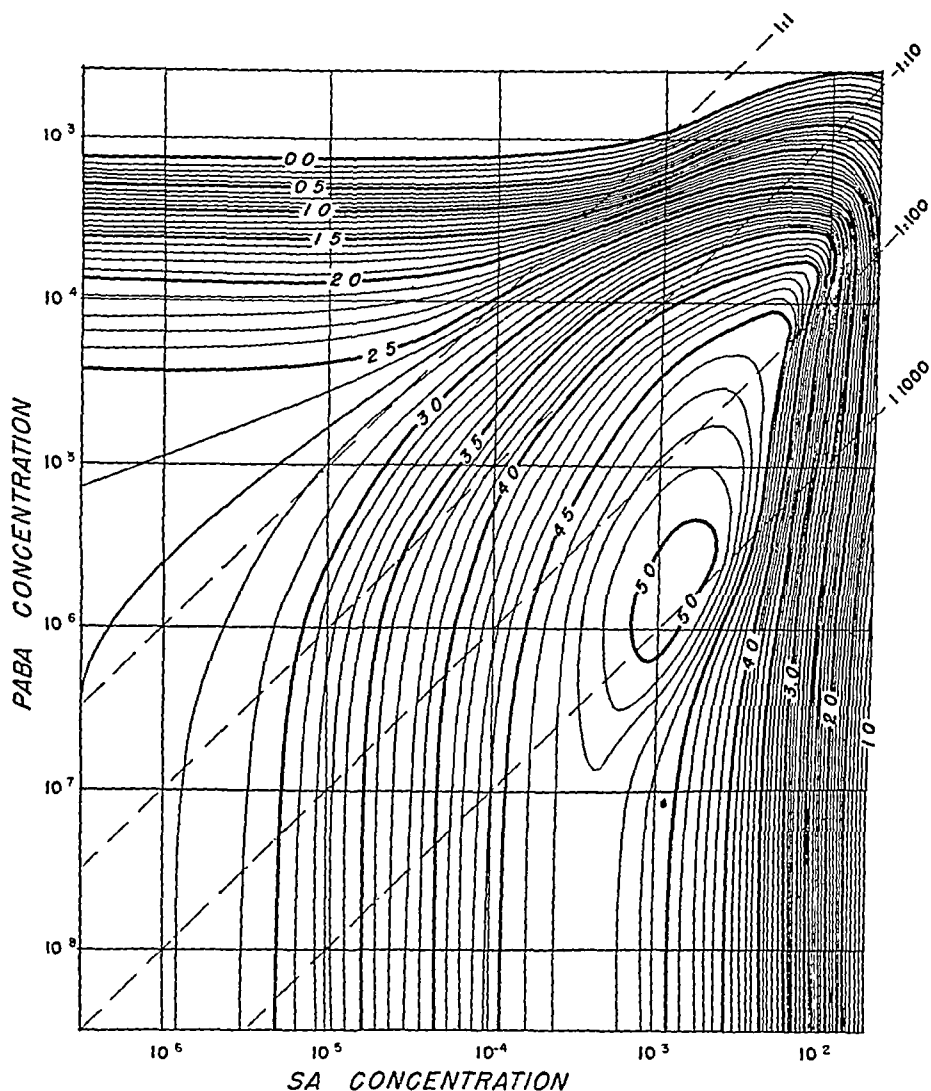
FIG 8 CONTOUR GRAPH SHOWING GROWTH RATES OF SFO STRAIN AT 30
        C IN THE PRESENCE OF VARYING AMOUNTS OF PABA AND SA
   See legend to figure 6   Based on 144 determinations at 96 different combinations of SA
and PABA concentrations

ber of nuclei carrying such mutations in the total nuclei tested from each culture
It follows that these mutations are not strictly reversions, but rather suppres-
sions of the effects of sfo by another gene
   Since the more rapid "reverted" growth is presumably always the result of an

altered genetic constitution, the rates obtaining before reversions occur are taken as characteristic of sfo

Competitive inhibition of growth by p-aminobenzoic acid   Under conditions which make sulfonamides essential for growth of the sulfonamide-requiring strain, PABA inhibits growth in very low concentrations   When grown at 35 C in the presence of optimal or suboptimal concentrations of SA, PABA will inhibit growth at concentrations as low as millionth molar (figure 6)   In the presence of in excess of SA, however, relatively small amounts of PABA are beneficial, though inhibition still occurs at higher concentrations   The stimulating effect of PABA at high SA concentration is principally in shortening the lag phase resulting from the toxicity of SA, as illustrated in figure 7, though the final growth rate may also be increased
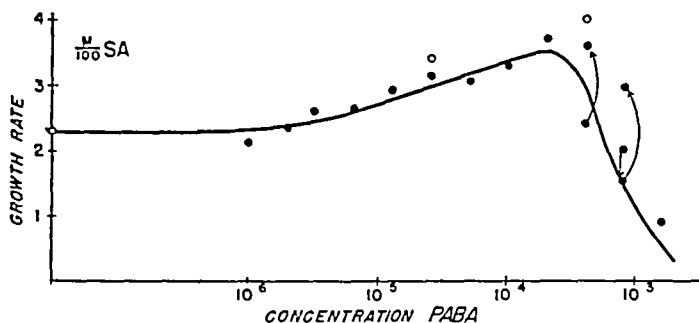
FIG 9 GROWTH RATES OF SFO STRAIN AT 30 C IN THE PRESENCE OF
M/100 SA AND VARYING MOLAR CONCENTRATIONS OF PABA
Growth rates expressed as millimeters per hour   Open circles represent data from one experiment, solid circles from another   Dots connected by arrows represent growth rates which changed during the course of growth down the tube (cf figures 5 and 7)

At lower temperatures, at which sulfonamides stimulate growth but are not essential for growth, the inhibitory effect of PABA is very much less (figure 8) Relatively high concentrations of PABA are necessary for growth inhibition, and the inhibiting concentration is relatively independent of the amount of SA present   On the other hand, PABA does interfere with growth stimulation by SA   Maximum response to SA occurs only when the molar ratio of PABA to SA is less than 1 to 100   Here again in the presence of excessive amounts of SA, PABA partially overcomes the inhibition caused by the SA (figure 9)

The simultaneous requirement of sulfanilamide and p-aminobenzoic acid by a double mutant   The sulfonamide-requiring strain was crossed to the pab strain of Tatum and Beadle (1942), which requires PABA for growth, and the double mutant (sfo pab) isolated [4]   At 35 C this double mutant requires both PABA and SA (figure 10)   Over most of the range of concentrations supporting growth

[4] Tatum and Beadle's pab strain 1633A was first crossed to wild-type strain E-5297a and the pab gene isolated free from an undesirable gene (temperature sensitive on lactose, etc , see Emerson and Cushing, 1946) as strain E-15835a, which was then crossed to sfo E-15172A The double mutant, sfo pab, was isolated from this cross in strains E-16608A and E-16613a

of the double mutant, a molar ratio of about 1 PABA to 1000 SA is most favorable   An excess of either analog is inhibitory in a competitive manner
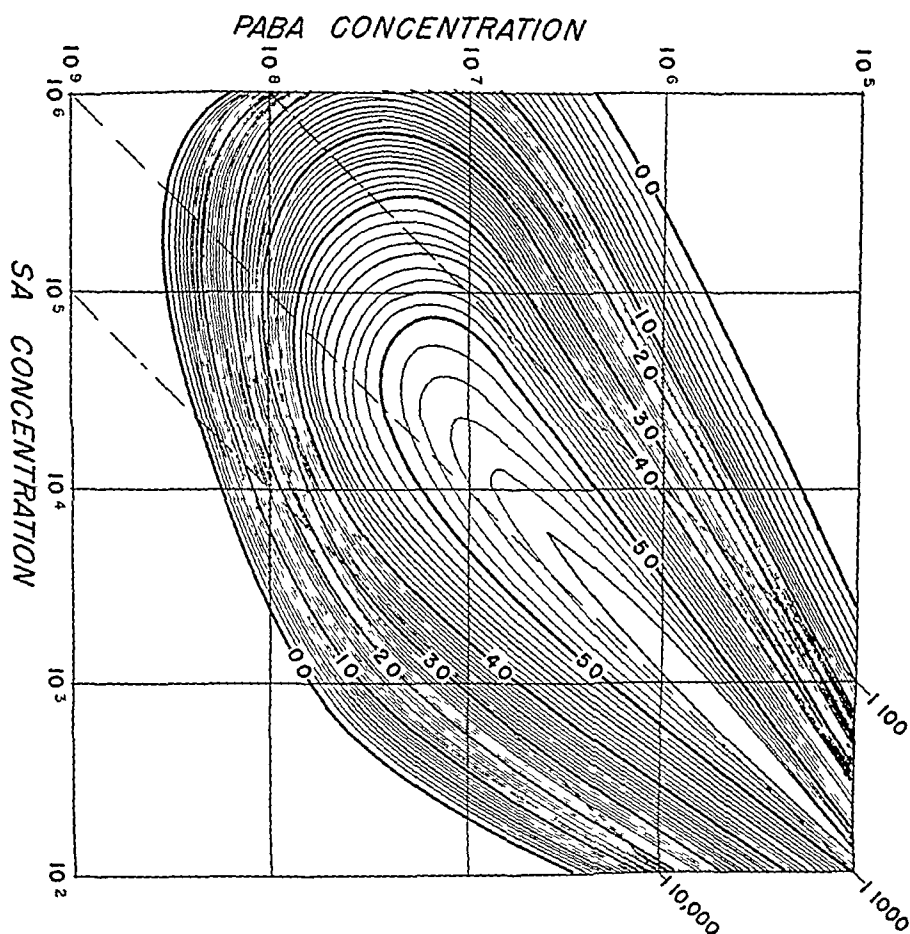
FIG 10 CONTOUR GRAPH SHOWING VARIATION IN GROWTH RATE WITH CHANGING
CONCENTRATIONS OF PABA AND SA OF THE DOUBLE MUTANT
(SFO PAB) AT 35 C
See legend to figure 6   Based on 84 determinations at 52 combinations of SA and PABA
concentrations

DISCUSSION

Sulfanilamide as a metabolite   Although growth responses by themselves do not prove that a substance found to be necessary for growth is actually used as a metabolite, the data just reported make it seem highly probable that sulfanilamide is so utilized by strain E-15172 (sfo)   In the first place, of the substances tested only sulfonamides were capable of supporting growth of the sfo strain at 35 C   Secondly, this strain does not produce excessive amounts of PABA and thus require sulfonamides as antagonists because (1) Inhibition of wild-type *Neurospora* by PABA is not antagonized by SA (Emerson and Cushing, 1946)

(2) The double mutant sfo pab cannot synthesize PABA and needs both SA and PABA for growth at 35 C   In the double mutant there can be no question of an overproduction of PAB A, yet sulfonamides are still required   Thirdly, the competitive inhibition of growth of the sfo strain by PABA suggests that the structurally similar SA is actually used as a metabolite

Sulfanilamide-p-aminobenzoic acid ratios   In most instances of competitive growth inhibition the molar ratio of inhibiting analog to metabolite is rather large   The competitive inhibition of wild-type *Neurospora* by SA is of this sort (Tatum and Beadle, 1942, Emerson and Cushing, 1946)   It is all the more striking, therefore, that in the PABA inhibition of the sulfonamide-requiring strain the ratio of inhibiting analog to "metabolite" is just reversed, being about 1 PABA to 100 SA

FIG 11 FORMAL SCHEME TO SUMMARIZE THE INTERPLAY OF MUTATIONS EFFECTING SA-PABA RELATIONSHIPS
Heavy arrows represent enzymatic reactions, breaks in arrows represent interruptions due to genetic blocks or to substrate inhibitions

Since the molar ratio of PABA to SA is the same regardless of which is the inhibiting analog, one is tempted to suggest that the same enzymatic reaction is involved in both cases   If two substrates (PABA and SA) compete for the same enzyme, and if both are transformed by that enzyme, the relative amounts of the two products resulting will depend upon the relative amounts of the two substrates   Then if wild-type requires one of these end products (Y in figure 11), and the sulfonamide-requiring mutant the other (Y'), the ratios of inhibiting analog to metabolite should be reversed as one metabolite is replaced by the other, just as reported above [5]
A formal scheme giving a pictorial summary of the interplay of the various

[5] Nearly everyone with whom I have discussed this case has suggested that the sulfonamide requirement of strain E-15172 might be accounted for by some such scheme   I believe that the particulars just outlined were first suggested by my collaborator Dr Marko Zalokar

mutations studied is given in figure 11   The aminobenzoicless mutant (pab) is known to interrupt the synthesis of PABA (Tatum and Beadle, 1942)   In the absence of PABA the gene pab is sometimes changed to $+^{pab}$ by reverse mutation, restoring the wild-type condition in which the synthesis of PABA continues normally

It is supposed that PABA takes part in more than one essential reaction (e g, with substances C and D in the diagram)   This would be in agreement with the observations of Lampen et al (1946), which suggest that PABA is concerned with three different sorts of syntheses   The inhibition of growth by SA is supposed to be due to substrate competition with PABA in one or more of these reactions Such SA inhibition can be lessened by nongenetic adaptation (Emerson and Cushing, 1946), or largely overcome by mutation to sulfanilamide tolerance (S-T)   Especially in the presence of PABA or sulfathiazole, reverse mutation changes S-T back to wild-type $(+^{S-T})$

The sulfonamide-requiring mutant, sfo, is shown as differing from wild-type by needing the end product Y' in place of Y   As illustrated, the double mutant pab sfo, requiring both PABA and SA for growth, needs X as well as Y'   On this supposition, SA would interfere with the production of X, PABA with the production of Y'   It is also possible that in place of X and Y' the double mutant needs Y and Y', say in approximately equal amounts   Again a balance between SA and PABA would be essential as the production of Y is inhibited by excess SA, of Y' by excess PABA

"Reversions" of the sfo mutant to growth resembling wild-type are due to "suppressor" mutations   These are mutations of a gene distinct from sfo which suppress the sulfonamide requirement characteristic of sfo

The scheme illustrated is meant simply as a convenient summary   Direct evidence of the role of SA in the metabolism of the sulfonamide-requiring mutant must await the chemical determination of the fate of SA in the organism

SUMMARY

Mutant strain E-15172 requires sulfonamides for growth at 35 C   At 30 C or lower sulfonamides are not strictly essential, but growth rates are depressed without them

At high temperatures (34 C or over) p-aminobenzoic acid inhibits growth of this strain at high dilutions (10^{-6} molar)   Growth inhibition by p-aminobenzoic acid is competitively antagonized by sulfanilamide   The ratio of p-aminobenzoic acid to sulfanilamide giving 50 per cent growth inhibition is about 1 100

A double mutant, carrying the gene for sulfonamide requirement and a gene for the failure of synthesis of p-aminobenzoic acid, requires both sulfonamides and p-aminobenzoic acid for growth   The molar ratio giving maximum growth at 35 C is about 1,000 sulfanilamide to 1 p-aminobenzoic acid

The possibility that sulfanilamide is utilized by strain E-15172 as a metabolite is discussed

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THE RELATION OF THE BACTERIAL PRODUCTION OF AMMONIA GAS TO THE GROWTH OF OTHER MICROORGANISMS

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In an experiment on the growth of *Neurospora crassa* a wide zone of inhibition was observed on the surface of agar surrounding a contaminating bacterial colony   The heat-resistant contaminant was isolated and labeled UB1   A few preliminary experiments indicated that the inhibition was of such a drastic nature as to warrant further investigation

Because the 24-hour growth of UB1, after being streaked across an agar plate, completely inhibited the growth of several strains of *Neurospora* which were inoculated about 3 or 4 cm away, it was suspected that the inhibiting factor may be a gas   In order to test this point the following experiment was performed   The base of a petri dish, 7 5 cm in diameter, was sterilized inside a 10-cm petri dish so that two surfaces of agar completely separated by glass could be prepared as shown in figure 1   Such surfaces were prepared with Fries medium (Ryan, Beadle, and Tatum, 1943) containing 0 5 per cent "casamino" acids and 2 per cent agar   A culture of UB1 was streaked on surface A and allowed to grow at 25 C for 40 hours   A wild-type strain of *Neurospora crassa*, 1A, was then inoculated onto the agar contained in the central petri dish (B)   Inhibition of mold growth was again observed, whereas control plates which had not been streaked with UB1 permitted luxuriant growth of *Neurospora*   Consequently, it was necessary to conclude that UB1 produced some substance which passed through the air over the edge of the inner petri plate

In a similar fashion it was shown that 8 other strains of *Neurospora*, including biochemical mutants, could be inhibited by UB1   In one experiment, although *Neurospora* was inhibited, the agar in the central plate (B) possessed deep agar colonies of a new contaminating bacterium   In a control plate, which had not been inoculated with either UB1 or *Neurospora*, no such colonies appeared   One of these new contaminating colonies was isolated (called UB2), and cultures of it were introduced into the agar in region (B) of the double petri plate   Here it grew only when strain UB1 was streaked around it in region A   Thus strain UB1, in addition to producing a gaseous inhibitor of *Neurospora*, produces a gaseous substance which enables the growth of UB2   These gases may, of course, be the same

The pH of the agar medium we had been using was 5 6 and optimum for the growth of *Neurospora*   It was observed in some control plates, which contained UB1 in region A but no other organism, that the pH of the central agar rose to between 7 and 8   Consequently, UB1 produces an alkaline gas which can raise the pH of the agar   This accounts for the inhibition of *Neurospora*, whose rate

of growth decreases rapidly with a change in pH from 6 5 to 8 0 (Ryan, Beadle, and Tatum, 1943)   In order to determine whether the change in pH of the agar was also responsible for the growth of UB2, this strain was grown in liquid 0 5 per cent casamino acids (Fries) at a series of different hydrogen ion concentrations   At pH's of 5, 6, and 9 it failed to grow, only pH's of 7 and 8 supported growth   This property accounts for the stimulation of the growth of UB2 by UB1, but the nature of the alkaline gas produced by the latter organisms remained to be determined

Since UB1 also changes the pH of the medium on which it grows to about 8, ammonia gas can be suspected as the agent   In the following experiments, the agar in region A of the double plates was brought to pH 7 because better growth of UB1 occurred there than at pH 5 6   Also for better growth a temperature of 37 C was used   All these experiments were controlled by double plates containing UB1 in region A alone, UB2 in region B alone, UB1 in region A and UB2 in region B, and, finally, no organisms in either region   It was found that raising the pH of the central agar in B to 7 by either sodium hydroxide or ammonium hydroxide resulted in the growth of UB2 in the absence of UB1   Moreover, when the central agar containing UB2 was left at pH 5 6 and ammonium
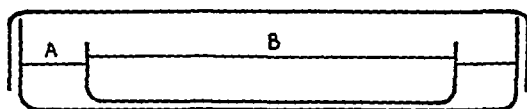
Fig 1 Optical Section of the Arrangement of one Petri Dish Inside Another,
Enabling the Preparation of Agar Surfaces Separated by Glass

hydroxide, instead of agar and UB1, was placed in region A, abundant growth ensued   Ammonia gas is therefore able to account for the experimental results

In order to demonstrate that UB1 produces gaseous ammonia, a culture was streaked on the agar in region A, while region B, instead of agar and UB2, contained 10 ml of 0 05 N sulfuric acid   After UB1 had grown for 24 hours, the sulfuric acid solution was examined for ammonia with Nessler's reagent   About 1 22 mg of ammonia was found, whereas about 1 3 mg are required to raise the pH of the buffered Fries medium from 5 6 to 7 5   It therefore appears that the gaseous ammonia produced by UB1 is sufficient to account both for the stimulation of UB2 and the inhibition of *Neurospora*

The ammonia produced by UB1 is undoubtedly derived from the amino acids in the casamino medium   UB1 will not use nitrate or ammonium ions as a nitrogen source, nor will UB2   The latter strain, at a pH of 5 6, will not use amino acids either, but will grow at that pH if tryptose is added   These two strains of bacteria differ in other respects, UB1 grows much more vigorously on all the media we have tried, it forms a pellicle on liquid, and its cells are shorter and thinner than those of UB2 (length of UB1, 2 to 2 5 μ, of UB2, 3 to 4 μ Both strains, however, consist of motile, aerobic, gram-positive rods which form central spores   These spores are, in both cases, very resistant to heat and will withstand boiling for 10 minutes   In addition, UB1 will grow on tryptose at

55 C, although apparently no better than at 37 C   The two strains have not been characterized further but appear to belong to the *Bacillus subtilis* group The production of ammonia by members of this group has been reported by Cook and Woolf (1928)

SUMMARY

A bacterial strain, secured from a plate contaminant, is able to produce ammonia gas in such amounts as to change the pH of buffered agar some distance away This behavior can result in the complete inhibition of the growth of the mold, *Neurospora crassa*, and, in addition, can enable the growth of a second strain of bacteria with a demanding pH requirement

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THE GROWTH AND PIGMENTATION OF ACTINOMYCES COELICOLOR AS AFFECTED BY CULTURAL CONDITIONS

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The use of characteristic pigments as criteria in species differentiation among the actinomycetes has been hampered by the comparative lack of knowledge of the conditions under which these pigments are produced   The striking red-blue indicator pigment of *Actinomyces coelicolor* (Muller) Lieske has been described (Conn, 1943) as a possible taxonomic character   The present investigation was designed to determine the cultural conditions which affect the formation of this pigment, with particular regard to carbon nutrition, nitrogen nutrition, and pH relations

MATERIALS AND METHODS

The culture of *A coelicolor* used has been described by one of us (Conn, 1943) as isolate B-3   The basal medium used throughout contained cp glucose, 10 g per L, Difco asparagine, 0 5 g per L, cp K_2HPO_4, 0 5 g per L, cp $MgSO_4$ $7H_2O$, 0 25 g per L, Difco yeast extract, 0 5 g per L, and a minor element mixture composed of salts of Zn, Fe, Cu, B, Mn, and Mo in the amounts recommended by Robbins and Ma (1942)   In this medium the sugar was found to be 85 to 90 per cent utilized in 18 days at 25 C, amounts of asparagine in excess of 0 5 g per L did not increase dry weight with glucose at 10 g per L   The organism was grown on the surface of 50 ml of liquid medium in 125-ml Erlenmeyer flasks   In still culture this and other actinomycetes make normal growth only on the surface, the flocculent subsurface mycelium so often noted in liquid cultures is characterized by a very slow and irregular growth rate and by poor sugar utilization

In experiments on carbon and nitrogen nutrition, replicate cultures were harvested at 6, 12, and 18 days after seeding, data from the last period only are reported   The dry weight was determined by drying the mycelium overnight at 70 C on tared filter paper   All pH measurements were made with the glass electrode

, To compare pigment production under different conditions, the filtered culture fluid was adjusted to pH 7 0 and diluted with 4 volumes of phosphate buffer (pH 6 98)   The intensity of pigmentation was then read in a Fisher electrophotometer against a similarly adjusted and diluted sterile medium, using a filter with peak transmittance at 650mμ

All cultures were run in triplicate   Dry weight determinations were made for each flask, other measurements were made on a pooled sample from the three replicates

CARBON AND NITROGEN NUTRITION

A condensed summary of data on the utilization of carbon and nitrogen sources is presented in table 1   Dry weight and pigment intensity were recalculated on the basis of glucose controls in the carbon source experiment, of asparagine controls in the nitrogen series

Except with organic acids, unavailability of a particular carbon source was evidenced not only by low relative growth but by a high final pH, resulting from the utilization of asparagine as a source of energy   Examples of this were the media having as carbon source sorbose, inulin, dulcitol, and sorbitol   With neutralized organic acids as energy sources, a rise in pH was in many cases evidence of utilization

Study of the growth data indicates that xylose, mannose, and glycerol were better carbon sources than glucose, arabinose proved to be much poorer, probably because of the production of acid   Sucrose was only slightly utilized, trehalose and lactose were slowly available   Among the organic acids, acetic, lactic, fumaric, succinic, malic, and gluconic acids were able to support growth in excess of that in the no-carbon control   Tartaric and citric acids did not support measurable mycelial growth, although the former was evidently attacked sufficiently to cause an increase in alkalinity

Pigment formation was proportional to mycelial growth with one general exception   when the final pH was either high or low, pigment intensity was not so great as would be expected from the dry weight   This is particularly notable among the organic acids, in the poorly buffered basal medium the utilization of neutralized lactic, fumaric, succinic, and malic acids resulted in final pH values above 8 7, and no pigment was formed   Utilization of acetate and gluconate was not accompanied by such extreme alkalinity, and some pigment developed A further exception to the general proportionality of growth and pigmentation was noted in glycerol media   The greater relative pigment intensity was not the result of reaction changes, it appears that glycerol specifically favors the production of pigment

Turning to the nitrogen nutrition data of table 1, it is evident that all of the four amino acids tested were utilizable, urea also proved an adequate source of nitrogen   On the other hand, neither nitrate nor ammonium salts supported growth comparable to that with asparagine, in the case of nitrate, the dry weight was no higher than in the glucose yeast extract control   With ammonium salts, the low final pH is the cause of poor growth, this acidity results undoubtedly from preferential absorption of the ammonium ion   Supplementary experiments with ammonium phosphate at several concentrations showed that satisfactory growth occurs if the pH can be held above 6 0   In a poorly buffered medium it is impossible to supply enough ammonium nitrogen for utilization of 1 per cent glucose without the development of too acid a reaction   Failure of growth in the nitrate medium may be associated with the observed heavy accumulation of nitrite

The several peptones tested supported excellent growth and pigmentation The latter fact shows that so-called "synthetic" media are not necessary for the

formation of the red-blue indicator pigment   As in the case of carbon sources, the intensity of pigmentation was roughly proportional to growth except in media with an unfavorable reaction   This is not always clear from the 18-day

TABLE 1

The utilization of carbon and nitrogen sources by A coelicolor

CARBON SOURCE*	CONCENTRATION	RELATIVE GROWTH†	RELATIVE PIGMENT INTENSITY†	FINAL pH	NITROGEN SOURCE‡	CONCENTRATION	RELATIVE GROWTH§	RELATIVE PIGMENT INTENSITY§	FINAL pH
	g/L					g/L			
None		17	0	8 4	None		30	32	6 7
d Glucose	10 0	100	100	7 2	l-Asparagine	0 50‖	100	100	6 7
d Mannose	10 0	202	200	7 0	Glycine	0 29‖	83	36	7 0
d Galactose	10 0	84	97	7 1	l-Leucine	1 00‖	76	36	7 0
d Fructose	10 0	79	96	7 0	l-Tryptophane	0 78‖	98	82	6 9
d Xylose	10 0	143	121	7 2	Urea	0 24‖	86	51	7 0
l Sorbose	10 0	26	0	8 5	NaNO₃	0 64‖	18	0	6 0
l Arabinose	10 0	65	46	5 2	(NH₄)₂HPO₄	0 50‖	57	0	5 7
Starch	10 0	107	87	7 0	Ammonium				
Inulin	10 0	32	0	8 5	acetate	0 58‖	18	0	5 6
Trehalose	10 0	90	38	8 1	Peptone	1 00	146	118	6 8
Cellobiose	10 0	81	95	6 7	Tryptone	1 00	91	106	7 0
Maltose	10 0	62	43	7 3	Casitone	1 00	116	100	7 0
Lactose	10 0	105	64	6 8	Pepticase	1 00	175	118	6 4
Sucrose	10 0	34	0	8 4	Casamino acids	1 00	116	129	6 6
Glycerol	10 0	135	170	6 6	Sodium				
Mannitol	10 0	82	88	7 1	caseinate	1 00	72	53	6 9
Dulcitol	10 0	20	0	8 6	Gelatin	1 00	106	29	6 4
Sorbitol	10 0	27	0	8 2	Egg albumin	1 00	44	35	6 0
Acetic acid	5 0	33	33	8 4					
Lactic acid	5 0	60	0	8 8					
Fumaric acid	5 0	69	0	9 1					
Succinic acid	5 0	47	0	8 9					
dl-Malic acid	10 0	60	0	8 9					
Tartaric acid	5 0	20	0	8 7					
Citric acid	5 0	24	0	8 2					
Gluconic acid	5 0	82	25	8 2					

* Basal medium (g/L) asparagine—0 5, yeast extract—0 5, K₂HPO₄—0 5, MgSO₄ 7H₂O —0 25, and minor elements
† Dry weight and pigment intensity of glucose control taken as 100
‡ Basal medium (g/L) glucose—10 0, yeast extract—0 5, K₂HPO₄—0 5, MgSO₄ 7H₂O— 0 25, and minor elements
§ Dry weight and pigment intensity of asparagine control taken as 100
‖ Total nitrogen, 0 106 g/L

data alone, thus, the relatively low pigment intensity of glycine and leucine media was associated with an acid reaction (about pH 6 0) earlier in the growth cycle The same was true of the gelatin medium

The data suggest that the nitrogen sources most favorable for growth and pigmentation—asparagine, tryptophane, and the peptones—are so not because

of the greater availability of nitrogen per se, but because the utilization of these materials is not accompanied by drastic pH changes   This, rather than specific nutrilite effects, may explain also the growth-promoting properties of yeast extract

If this is indeed the case, an excess of any nitrogen source, if unbalanced by an increase in the glucose level, should cause a reaction unfavorable for pigment formation   The experiment summarized in table 2 affords confirmation of this hypothesis   Failure of pigment to develop in high peptone media was associated with a high pH, but there was no measurable reduction in growth   Experiments not reported in detail showed that the deleterious effect of high peptone levels on chromogenesis can be eliminated by increasing the concentration of glucose

TABLE 2

*The effect of peptone concentration on growth and pigmentation of A coelicolor**

NITROGEN SOURCE	CONCEN TRATION	pH		DRY WEIGHT	RESIDUAL SUGAR	COLOR†	PIGMENT INTENSITY
		Initial	Final				
	g/L			mg	mg/100 ml		
None		6 62	7 01	36 2	306 4	P	12 5
Asparagine	0 5	6 94	6 90	68 3	138 1	P	26 0
Peptone	0 5	6 86	6 99	54 0	303 2	P	23 0
Peptone	1 0	6 85	6 98	87 2	33 4	P	26 8
Peptone	2 0	6 88	7 69	83 1	8 4	B	39 3
Peptone	5 0	6 84	8 46	81 7	13 9	0	

* Basal medium (g per L)   glucose—10 0, yeast extract—0 5, K_2HPO_4—0 5, $MgSO_4$ $7H_2O$—0 25, and minor elements   Duration of experiment 18 days
† 0—none, P—purple, B—blue

From these considerations it follows that the apparent utilization of a given source of nitrogen in part depends on the nature of the carbon source used and its concentration, since these factors affect the pH of the medium

THE INFLUENCE OF PH ON GROWTH AND PIGMENTATION

Changes in reaction have been postulated as the explanation of several phenomena of nutrition, especially with regard to the utilization of organic acids, peptone, and ammonium salts   An experiment, summarized in table 3, tested the effect of variations in the initial pH of the culture medium on growth and pigment formation

Growth occurred in media adjusted initially to pH 5 0 to 10 9, with the maximum dry weights at pH 6 9 to 7 7   The range in which pigment formed was somewhat narrower, pH 6 0 to 9 9, the optimum for pigment intensity being pH 7 3 to 7 7   It is evident that the organism is able substantially to lower the pH of alkaline media when glucose is the source of carbon, in order to obtain a final pH comparable to that attained in high peptone media, the medium had to be initially at pH 11 0   The difference between the pH limits of growth and

those of pigment formation explains the failure of pigmentation to accompany growth in media which become acid or alkaline during metabolism

TABLE 3

*The influence of the pH of the medium on growth and pigment prouction of A coelicolor**

MEDIUM	pH		DRY WEIGHT	COLOR‡	PIGMENT INTENSITY
	Initial†	Final			
			mg		
A	3 70	3 63	—0 2	0	
B	4 21	4 24	0 3	0	
C	4 9S	4 92	27 5	0	
D	6 00	6 30	65 0	R	12 5
E	6 90	6 59	89 7	P	23 6
F	7 31	6 97	87 5	P	32 9
G	7 70	6 97	82 0	P	34 9
H	S 0S	7 40	67 0	B	19 7
I	S 30	7 46	61 5	B	22 9
J	9 00	7 50	52 0	B	21 2
K	9 42	7 81	62 5	B	18 8
L	9 92	7 94	54 7	B	8 9
M	10 92	8 61	30 0	0	
N	11 20	11 06	0 3	0	

* Basal medium (g/L) glucose—10 0, asparagine—0 5, yeast extract—0 5, K_2HPO_4—0.5, $MgSO_4$ $7H_2O$—0 25, and minor elements   Glucose added aseptically after sterilization Duration of experiment 18 days
† Adjusted with HCl or NaOH, pH measured after sterilization
‡ 0—none, R—red, P—purple, B—blue

DISCUSSION

The controlling factor in the production of pigment by a vigorously growing culture of *A coelicolor* is the reaction of the medium   Within the range which permits chromogenesis, the actual color of the pigment is again determined by pH   Of the variety of carbon and nitrogen sources tested, there was none which supported growth but not chromogenesis except those compounds the presence of which or the utilization of which caused the pH to drop below about 6 0 or to rise above about 8 5

Within the pH range favorable for chromogenesis there is a discernible effect of reaction on the amount of pigment formed   The pH range for optimum production of pigment is somewhat narrower than the range of maximum mycelial growth   For this reason it is possible to effect changes in the amount of color without changing the final dry weight of the culture

The possible taxonomic value of the pigments of actinomycetes has been discussed elsewhere (Conn and Conn, 1941, Conn, 1943)   The present work emphasizes the need for careful control of certain environmental factors, and demonstrates that such control makes it possible to obtain reproducible results Parenthetically, it may be mentioned that the strain of *A coelicolor* used has been

carried in culture for 8 years with no detectable change in the pigment or in other characters   ;

SUMMARY

The growth of *Actinomyces* (*Streptomyces*) *coelicolor* Muller in surface culture has been studied with particular reference to the formation of the pigment characteristic of this species

In a survey of carbon sources, mannose, xylose, and glycerol were found to support the heaviest mycelial growth   The organism is able to utilize a wide range of sugars, polyatomic alcohols, and organic acids   Compounds not utilized included sorbose, inulin, sorbitol, dulcitol, tartaric acid, and citric acid, sucrose is only slightly utilized

Satisfactory nitrogen sources for growth include several amino acids and peptones, urea, casein, and gelatin   Ammonium salts of weak acids support normal growth only in a buffered medium, in a poorly buffered medium the acidity arising from preferential absorption of the ammonium ion interferes with growth Nitrate is absorbed but undetermined secondary effects make it unsuitable under the conditions tested

The optimum pH for growth is pH 6 9 to 7 7, the lower limit of growth is pH 4 2 to 5 0, the upper limit pH 11   Pigment is formed in media having an initial pH of 6 0 to 9 9, the optimum being pH 7 3 to 7 7

Regardless of the specific compounds used to supply carbon and nitrogen, pigment develops in any medium able to support mycelial growth, provided that the course of metabolism does not shift the final reaction to either side of the range pH 6 0 to 8 5   Any medium, "synthetic" or not, which supports growth without drastic pH changes also supports chromogenesis

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BIOTIN AND THE SYNTHESIS OF ASPARTIC ACID BY MICROORGANISMS

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Considerable evidence is accumulating concerning the role of growth factors in the metabolism of amino acids by microorganisms   Koser, Wright, and Dorfman (1942) demonstrated a relationship between aspartic acid and biotin in that aspartic acid can serve as a partial substitute for biotin in the growth of *Torula cremoris*   Pantothenic acid influences the synthesis of tryptophane by *Staphylococcus aureus* (Sevag and Green, 1944)   A member of the vitamin B₆ group, pyridoxal phosphate, is the coenzyme for the decarboxylation of tyrosine, lysine, arginine, and other amino acids (Gale and Epps, 1944, Gunsalus, Bellamy, and Umbreit, 1944, Baddiley and Gale, 1945, Umbreit and Gunsalus, 1945)   Pyridoxamine and pyridoxal are involved in the synthesis of lysine, threonine, and alanine by lactic acid bacteria (Stokes and Gunness, 1945)   A combination of vitamin B₆ and CO_2 apparently promotes synthesis of arginine, phenylalanine, and tyrosine by *Lactobacillus arabinosus* (Lyman et al , 1947)

Few data have been available concerning the specific role of biotin in the growth of microorganisms   That biotin must play an important metabolic role is indicated by the need for biotin by many microorganisms for growth, its wide distribution in cells, and its great activity per unit weight   The data presented below demonstrate that biotin is involved in the synthesis of aspartic acid by microorganisms   A preliminary report of this work has been published (Stokes, Larsen, and Gunness, 1947)

METHODS

Stab cultures of the bacteria were carried in a medium of the following composition   1 g of glucose, 0 5 g of Difco peptone, 0 6 g of anhydrous sodium acetate, salts A and B in half the concentration given in table 1, and 1 5 g of agar per 100 ml of medium, at pH 6 8   Inocula for the experiments were prepared by subculturing from stab cultures into a liquid medium of the same composition as that given above   After incubation for 16 to 24 hours at 37 C, the cells of the broth cultures were centrifuged, washed with water, and suspended in 100 ml of water   One drop of this suspension served to inoculate each tube in an experiment   The basal medium (table 1) was prepared as described previously (Stokes and Gunness, 1945) and distributed in 5-ml quantities in 22-by-150-mm tubes

After addition of the experimental compounds, the volume in the tubes was brought to 10 ml with water prior to sterilization by autoclaving   Unless indicated otherwise, cultures of *Streptococcus faecalis* R were incubated for 40 hours and the remaining organisms for 64 hours at 37 C, at which times maximum

acid production has occurred   Lactic acid was determined by titration with alkali using bromthymol blue as the indicator   *S faecalis* R cultures were titrated with 05 N NaOH and the other bacteria with 0 1 N NaOH   *S faecalis* forms less acid than the lactobacilli in the basal medium employed   The titrations were made directly in the culture tubes   Growth is usually expressed in terms of the amount of acid formed in the cultures since the latter can be easily measured quantitatively   Synthetic *dl*-aspartic acid was used in all experiments   The biotin was *d*-biotin obtained from synthetic *dl*-biotin   Additional details of methods are described later

<p style="text-align:center">TABLE 1</p>
<p style="text-align:center">Basal medium</p>

dl-Leucine	100 mg	Sodium acetate (anhydrous)	3 g
dl-Isoleucine	100 mg	Adenine	5 mg
dl-Valine	100 mg	Guanine	5 mg
l(−)-Cystine	100 mg	Uracil	5 mg
dl-Methionine	100 mg	Pantothenic acid	100 μg
dl-Tryptophane	200 mg	Riboflavin	100 μg
l(−)-Tyrosine	100 mg	Thiamine HCl	100 μg
dl-Phenylalanine	100 mg	Nicotinic acid	100 μg
dl-Glutamic acid	100 mg	Pyridoxamine	200 μg
dl-Threonine	100 mg	*p*-Aminobenzoic acid	20 μg
dl-Alanine	100 mg	Biotin	0 1μg
dl-Aspartic acid	100 mg	Folic acid*	1 0μg
l(+)-Lysine	50 mg	Salts A	
l(+)-Arginine	100 mg	K$_2$HPO$_4$	250 mg
l(+)-Histidine	100 mg	KH$_2$PO$_4$	250 mg
dl-Serine	100 mg	Salts B	
l(−)-Proline	100 mg	MgSO$_4$ 7H$_2$O	100 mg
l(−)-Hydroxyproline	100 mg	NaCl	5 mg
dl-Norleucine	100 mg	FeSO$_4$ 7H$_2$O	5 mg
Glycine	100 mg	MnSO$_4$ 4H$_2$O	5 mg
Glucose	5 g	Adjust to pH 6 8	
		Add distilled H$_2$O to	250 cc

* Obtainable from Dr R J Williams, University of Texas, Austin, Texas, pteroyl glutamic acid may also be used

Equivalent to 1 0 μg of material of "potency 40,000" or 1 0 μg of pteroyl glutamic acid

<p style="text-align:center">EXPERIMENTS</p>

In preliminary experiments designed to extend the basic microbiological assay method for the ten essential amino acids (Stokes, Gunness, Dwyer, and Caswell, 1945) to include the assay of aspartic acid, poor agreement of values at different levels of impure proteins was noted   The test organism was *Streptococcus faecalis* R, which in the usual synthetic media (table 1) requires aspartic acid for growth   An attempt was made to improve the basal medium by increasing the content of vitamins and the purine and pyrimidine bases fivefold   Surprisingly, this change caused almost maximum growth and lactic acid formation of *S*

faccalis in the blank tubes which contained no aspartic acid   It appeared, therefore, that the increase in growth factor supplements stimulated synthesis of aspartic acid by *S faccalis*   Fractionation of the growth factor mixture demonstrated that the increase in biotin alone was responsible for the growth of *S faccalis* in the absence of aspartic acid (table 2)   Increases in adenine, guanine, uracil, riboflavin, pantothenic acid, thiamine, nicotinic acid, *p*-aminobenzoic acid, pyridoxamine, and folic acid were ineffective in supporting appreciable growth in the absence of aspartic acid

The ability of biotin to substitute for aspartic acid is not confined to *S faecalis* R   A survey of eight additional aspartic-acid-requiring bacteria revealed that, with the exception of the heterofermentative *Leuconostoc mesenteroides* P-60,

TABLE 2

Effect of increased concentrations of growth factors on development of Streptococcus faccalis R in the absence of aspartic acid

ADDENDUM*	GROWTH†	ML 0 05 N LACTIC ACID FORMED PER 10 ML OF MEDIUM†
Nil	+	2 1
Aspartic acid, 0 5 mg ,	++++	12 7
Fivefold increase in		
All vitamins + adenine, guanine, uracil	++++	11 4
Adenine, guanine, uracil	+	1 9
Riboflavin	+	2 2
Pantothenic acid	+	2 1
Thiamine	+	2 1
Nicotinic acid	+	2 0
Biotin	++++	10 2
p Aminobenzoic acid	+	2 1
Pyridoxamine	+	2 0
Folic acid	+	2 0

* Added to the basal medium (table 1) from which aspartic acid was omitted
† After incubation at 37 C for 40 hr

addition of excess biotin to the basal medium resulted in full or almost full growth, as measured by acid production, of all strains of streptococci and lactobacilli tested in the absence of aspartic acid (table 3)   For *Streptococcus faecalis* 10C1 and F24 and for *Streptococcus zymogenes* 5C1, 0 5 millimicrograms of biotin were sufficient to permit considerable growth in the absence of aspartic acid, although the stimulatory effect of additional biotin is clearly evident

In figure 1 it can be seen that if a production of 6 ml of acid is used as a point of reference, it is necessary to supply the *Lactobacillus casei* strains with 3 to 5 times, and *L arabinosus* with 2 7 times, as much biotin for growth in the absence of aspartic acid as when aspartic acid is present   Similar ratios were obtained for the other bacteria listed in table 3   It is also evident from the graph that the lactobacilli require biotin for growth even when liberally supplied with aspartic acid, a fact which indicates that biotin is required for metabolic func-

tions other than those concerned with synthesis of aspartic acid   From the quantitative biotin ratios given above, it appears that much more biotin is neces

TABLE 3

Substitution of biotin for aspartic acid in the growth (acid formation) of various aspartic-acid-requiring bacteria

MICROORGANISM	0 5 MILLIMICROGRAMS BIOTIN PER 10 ML MEDIUM		20 MILLIMICROGRAMS BIOTIN PER 10 ML MEDIUM	
	No aspartic acid	2 mg dl aspartic acid	No aspartic acid	2 mg dl aspartic acid
	*ml acid formed per 100 ml medium**			
Streptococcus faecalis R	0 8	11 1	8 9	12 9
Streptococcus faecalis 10Cl	5 9	9 8	10 7	11 1
Streptococcus faecalis F24	4 7	9 8	11 0	12 1
Streptococcus durans 98A	3 1	11 1	10 8	11 5
Streptococcus zymogenes 5Cl	4 7	9 8	11 0	12 1
Lactobacillus casei LD5†	1 2	8 5	8 8	10 5
Lactobacillus casei	1 6	9 0	8 8	11 7
Lactobacillus arabinosus 17-5	0 7	9 2	11 0	11 5
Leuconostoc mesenteroides P-60	0 9	13 4	0 8	17 3

* The lactobacillus cultures were titrated with 0 1 N NaOH and the remaining cultures with 0 05 N NaOH after 3 days' incubation at 37 C

† Formerly known as *Lactobacillus delbrückii* LD5 but recently identified as a strain of *Lactobacillus casei* (Rogosa, 1946)
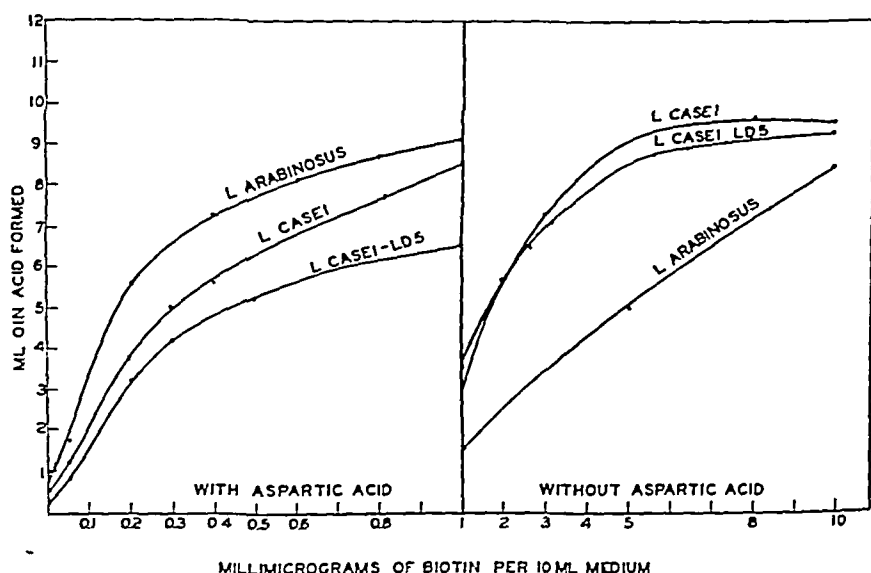
FIG 1 QUANTITIES OF BIOTIN REQUIRED FOR GROWTH OF LACTOBACILLI WITH AND WITHOUT ASPARTIC ACID

sary for synthesis of aspartic acid than for the other function or functions of biotin   The need of the bacteria for biotin in the presence of aspartic acid elimi

nates the possibility that all of the foregoing results could be explained by assuming that the bacteria do not require aspartic acid but that growth with aspartic acid is due to biotin present as an impurity in the aspartic acid

The biotin-aspartic-acid relationship is very specific As previously indicated (table 2) only biotin of the vitamins tested stimulated growth in the absence of aspartic acid Also, although S faecalis R, L arabinosus, and L casei require leucine, isoleucine, valine, cystine, methionine, tryptophane, tyrosine,

TABLE 4

Influence of biotin on the amino acid requirements of lactic acid bacteria

AMINO ACID OMITTED	STREPTOCOCCUS FAECALIS R		LACTOBACILLUS ARABINOSUS 17 5		LACTOBACILLUS CASEI	
	0 0005 μg Biotin	0 1 μg Biotin	0 0005 μg Biotin	0 1 μg Biotin	0 0005 μg Biotin	0 1 μg Biotin
	ml acid formed per 10 ml medium					
None	12 4	13 8	9 6	12 5	8 7	11 3
Leucine	0 8	1 2	0 6	0 6	0 5	0 6
Isoleucine	1 6	2 1	0 8	0 6	0 6	0 7
Valine	0 7	1 4	0 7	0 8	0 6	0 5
Cystine	9 2	11 3	6 3	8 9	0 7	0 7
Methionine	1 6	1 7	0 7	1 2	6 1	6 1
Tryptophane	1 1	1 6	0 6	0 8	0 7	0 7
Tyrosine	2 4	1 8	1 1	1 0	1 2	1 2
Phenylalanine	9 9	10 1	0 5	0 6	0 6	0 7
Glutamic acid	0 6	1 2	0 5	0 6	0 6	0 7
Threonine	0 6	0 7	8 8	9 8	8 6	9 7
Alanine	7 1	6 7	9 1	10 0	8 8	9 8
Aspartic acid	0 9	12 8	1 0	11 6	2 6	7 8
Lysine	1 0	0 8	8 8	10 2	8 5	9 6
Arginine	1 1	1 2	1 1	1 0	0 9	1 0
Histidine	1 6	1 7	8 4	9 6	8 3	8 8
Serine	7 0	6 9	8 3	9 4	0 4	0 6
Proline	12 3	13 1	8 8	10 6	8 6	9 0
Hydroxyproline	12 1	14 0	8 9	9 9	8 9	9 2
Norleucine	11 2	12 5	8 2	10 2	8 3	8 8
Glycine	7 9	9 8	7 7	8 1	7 8	8 2

phenylalanine, glutamic acid, threonine, lysine, arginine, histidine, and serine for growth in addition to aspartic acid, the requirement for only aspartic acid is eliminated by the use of excess biotin in the medium (table 4)

So far it has been assumed that the ability of biotin to substitute for aspartic acid in the nutrition of the bacteria indicates that biotin is involved in the synthesis of that amino acid However, since it has been shown, apparently, that the proteins of certain algae are lacking in lysine, tyrosine, arginine, and methionine (Mazur and Clarke, 1938, 1942), it seemed necessary to prove that the bacterial cells grown with excess biotin in place of aspartic acid actually

3 Cysteic acid $+$ oxalacetic acid $\rightarrow$ aspartic acid $+$ sulfapyruvic acid (Cohen and Hekhus, 1941)

4 Succinic acid $\xrightarrow{-2H}$ fumaric acid $\xrightarrow{+H_2O}$ malic acid $\xrightarrow{-2H}$ oxalacetic acid (Harrow, 1940), followed by reaction (1) to give aspartic acid

5 Fumaric acid $+$ NH_3 $\rightarrow$ aspartic acid (Quastel and Woolf, 1926)

6 Pyruvic acid $+$ CO_2 $\rightarrow$ oxalacetic acid (Krampitz, Wood, and Werkman, 1943), followed by reaction (1) to give aspartic acid

It is evident that with the exception of reaction (5), transamination is directly or indirectly involved in all of them

In a typical experiment *L arabinosus* was grown in 250-ml Erlenmeyer flasks containing 100 ml of the basal medium (table 1)   The biotin content, however, was reduced to the very small quantity of 05 millimicrogram per 10 ml of medium, and 200 μg of oleic acid per 10 ml were added as a substitute for the remainder of the required biotin (Williams and Fieger, 1946)   The medium was adjusted to pH 5 6   Cells harvested from such a medium are essentially free from biotin   After incubation for 3 days at 37 C, the cells were collected by centrifugation, washed once with M/15 phosphate buffer at pH 7, and resuspended in sufficient buffer of the same type to give a galvanometer deflection of 5 on the Evelyn photoelectric colorimeter at 520 millimicrons wave length   Four-ml aliquots of cell suspension were mixed in test tubes (22 by 150 mm) with 10 mg each of the compounds shown in table 6 except that 20 mg of *dl*-alanine were used   To one of duplicate sets, 5 μg of biotin were added   Where necessary, the pH of the suspensions was adjusted to pH 7 and the volume to 5 ml   The thoroughly shaken tubes were stoppered and incubated overnight, for approximately 18 hours at 37 C   After incubation, the aspartic acid in the suspensions, cells plus fluid, was determined by quantitative assay with *Leuconostoc mesenteroides* (Hac and Snell, 1945) employing the medium shown in table 1 and a total assay volume of 1 0 ml   Titrations were made with 0 01 N NaOH   In this way as little as 2 μg to 10 μg of aspartic acid per ml of suspension could be readily measured (figure 2)

It is evident from table 6 that resting cells of *L arabinosus* form aspartic acid from glutamic acid, alanine, or cysteic acid plus oxalacetic acid   Malic and fumaric acids and to a lesser extent succinic acid can substitute for oxalacetic acid, presumably because they are converted to oxalacetic acid by the resting cells   However, all of these reactions proceed as well without biotin as with it   This clearly indicates that biotin is not involved in any of these reactions   The cells in this particular experiment contained less than 04 millimicrograms of biotin per ml of suspension as measured microbiologically (Wright and Skeggs, 1944) on acid-hydrolyzed cells   Therefore, the possibility of carry-over of significant amounts of biotin by the cells is eliminated   Glutamine can replace glutamic acid to give aspartic acid with either malic, fumaric, or succinic acid   Similar results were obtained with *L arabinosus* cells grown in media containing excess biotin and no aspartic acid, and also in media with vitamin-free casein hydrolyzate, as a substitute for all of the amino acids except cystine and trypto-

phane, plus either oleic acid or sufficient biotin for half-maximum growth (0 2 millimicrograms per 10 ml) to reduce carry-over of biotin from the medium by the cells   In general, resting cells of *S faccalis* R and *L casci* gave results similar

TABLE 6

Formation of aspartic acid by cell suspensions of Lactobacillus arabinosus

CELLS PLUS ADDENDA	NO BIOTIN	PLUS BIOTIN
	Aspartic acid	
	micrograms per ml of suspension	
Nil	3	3
Glutamic acid + oxalacetic acid*	28	26
Glutamic acid + malic acid	108	104
Glutamic acid + fumaric acid	140	148
Glutamic acid + succinic acid	14	14
Alanine + oxalacetic acid	14	14
Cysteic acid + oxalacetic acid	13	17

* Ninety-two per cent pure, kindly supplied by Dr L O Krampitz
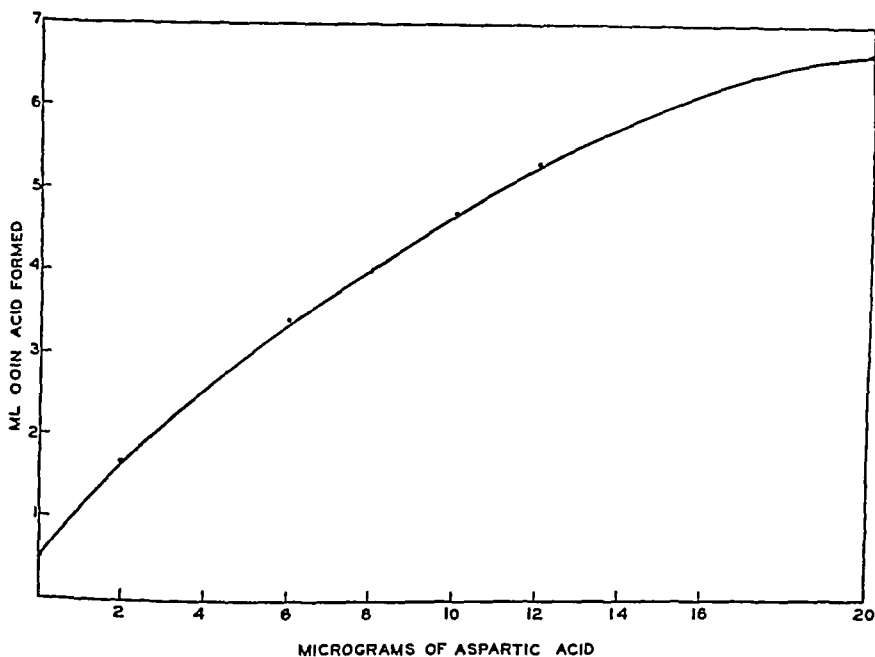
FIG 2 RESPONSE OF LEUCONOSTOC MESENTEROIDES TO *l*-ASPARTIC ACID

to those obtained with *L arabinosus*   Indirect evidence that biotin does not catalyze any of the reactions in table 6 is the fact that resting cells of *L mesenteroides,* whose requirement for aspartic acid is not influenced by biotin, also produce aspartic acid under these conditions

No evidence could be obtained with *L arabinosus* for the formation of aspartic acid by the direct amination of fumaric acid   Similarly negative results were obtained with malic or succinic acid and $(NH_4)_2SO_4$ In this connection, however, it may be significant that biotin-deficient yeast cells are markedly stimulated by biotin to take up ammonia (Winzler, Burk, and duVigneaud, 1944)   Our negative results may merely indicate that the proper physiological conditions were not provided in the resting cell suspension experiments

Also, no aspartic acid was formed in cell suspensions of *L arabinosus* supplied with glutamic acid, plus pyruvic acid and either $NaHCO_3$ or CO_2 gas as a source of carbon dioxide   These negative results were not altered by the addition of thiamine, pyridoxamine, *p*-aminobenzoic acid, riboflavin, pantothenic acid, nicotinic acid, folic acid, glucose, and adenosine triphosphate to the suspensions,

TABLE 7

Stimulation of growth (acid formation) of lactic acid bacteria by oxalacetic acid in aspartic-acid-free medium

COMPOUND ADDED	L CASEI	S FAECALIS	L ARABINOSUS	L MESENTEROIDES
*Per 10 ml medium**	*ml 0.1 N acid formed per 10 ml medium*			
Nil	4 6	0 8	3 3	0 5
dl-Aspartic acid, 2 mg	9 6	6 8	9 5	8 7
Biotin, 0 1 µg	9 6	6 1	10 0	0 5
Oxalacetic acid,† 1 mg	7 9	0 8	4 1	0 5
Oxalacetic acid, 5 mg	10 0	0 8	6 3	0 5
Oxalacetic acid, 25 mg	9 4	0 7	7 5	0 5

* Basal medium contained 0 8 millimicrograms of biotin and no aspartic acid
† Sterilized by filtration

by varying the pH of the suspension from pH 6 to pH 8, nor by the use of acetone-dried cells possibly to increase permeability of the cells to adenosine triphosphate   The acetone-dried cells readily formed aspartic acid when mixed with glutamic and oxalacetic acids

A suggestion that biotin may be concerned with the formation of oxalacetate was obtained from growth experiments in which for *L casei* and *L arabinosus* but not for *S daccalis* R oxalacetic acid partially replaced biotin in aspartic-acid-deficient media (table 7)   The possibility that the activity of the oxalacetic acid was due to impurities of biotin or aspartic acid was ruled out by assay of the preparation for these two components

SUMMARY

Biotin can completely substitute for aspartic acid in the growth of *Lactobacillus arabinosus*, *Streptococcus faecalis*, and related organisms   The biotin aspartic-acid relationship is specific, riboflavin, pantothenic acid, thiamine, *p*-aminobenzoic acid, and pyridoxamine cannot replace biotin, nor can biotin substitute for 14 amino acids other than aspartic acid which are required for growth   Cells grown with biotin contain as much aspartic acid as those grown

with aspartic acid It is concluded that biotin participates in the synthesis of aspartic acid Although resting cell suspensions of *Lactobacillus arabinosus* can form aspartic acid by typical transamination reactions, the presence of biotin is not required for such reactions It has not been possible to determine the specific aspartic-acid-forming reaction catalyzed by biotin

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THE MORPHOLOGY OF THE L₁ OF KLIENEBERGER AND ITS RELATIONSHIP TO STREPTOBACILLUS MONILIFORMIS[1]

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In 1945 the author published observations concerning the morphology of the pleuropneumonia group of organisms (Dienes, 1945). The methods used for the study of these organisms have been applied in the present work to *Streptobacillus moniliformis* and its L₁ variant. These organisms have been studied previously (Dienes, 1942), but improvements of technique in the meantime have made it possible to observe more clearly the form of the individual organisms and their derivation from each other. The properties of the L₁ and its connection with the bacillus challenge several accepted concepts in bacteriology. Most authors, taking into consideration that the L₁ originates from the bacillus, that it is serologically similar, and that under appropriate conditions it reproduces the bacillus, accept the conclusion that these two organisms, so different in appearance, are growth forms of the same organism. Klieneberger (1942) recently reaffirmed her objections to this conclusion, her chief objection is that the morphology of the L₁ is different from that of bacteria. Hence more accurate information should bring into agreement the different views on its nature. Such information is needed also as a basis to establish the biological significance of these peculiar bacterial forms.

The difficulties which prevented for a long time an adequate concept of the morphology of the pleuropneumonia group of organisms are present in L₁ to an even greater extent. The organisms are exceedingly fragile and soft, they adhere firmly to each other, and the colonies grow into the agar. In broth, soft dense clumps are formed. The best way to overcome these difficulties, as in the case of the pleuropneumonia group, has been in the staining of the colonies on the agar. By studying several strains in various stages of development it was possible to observe the forms which comprise the colonies. The agar fixation method (Klieneberger and Smiles, 1942), which gives excellent preparations with many strains of the pleuropneumonia group, is not applicable to small L₁ colonies, because they either do not adhere to the glass or, if they adhere, they are in dense clumps. Only the surface of well-developed colonies adheres to the glass, and some of the most successful photographs, both of the small and large forms of the organisms, were made from such preparations. This method alone is not, however, sufficient for the study of the cultures because it does not allow one to
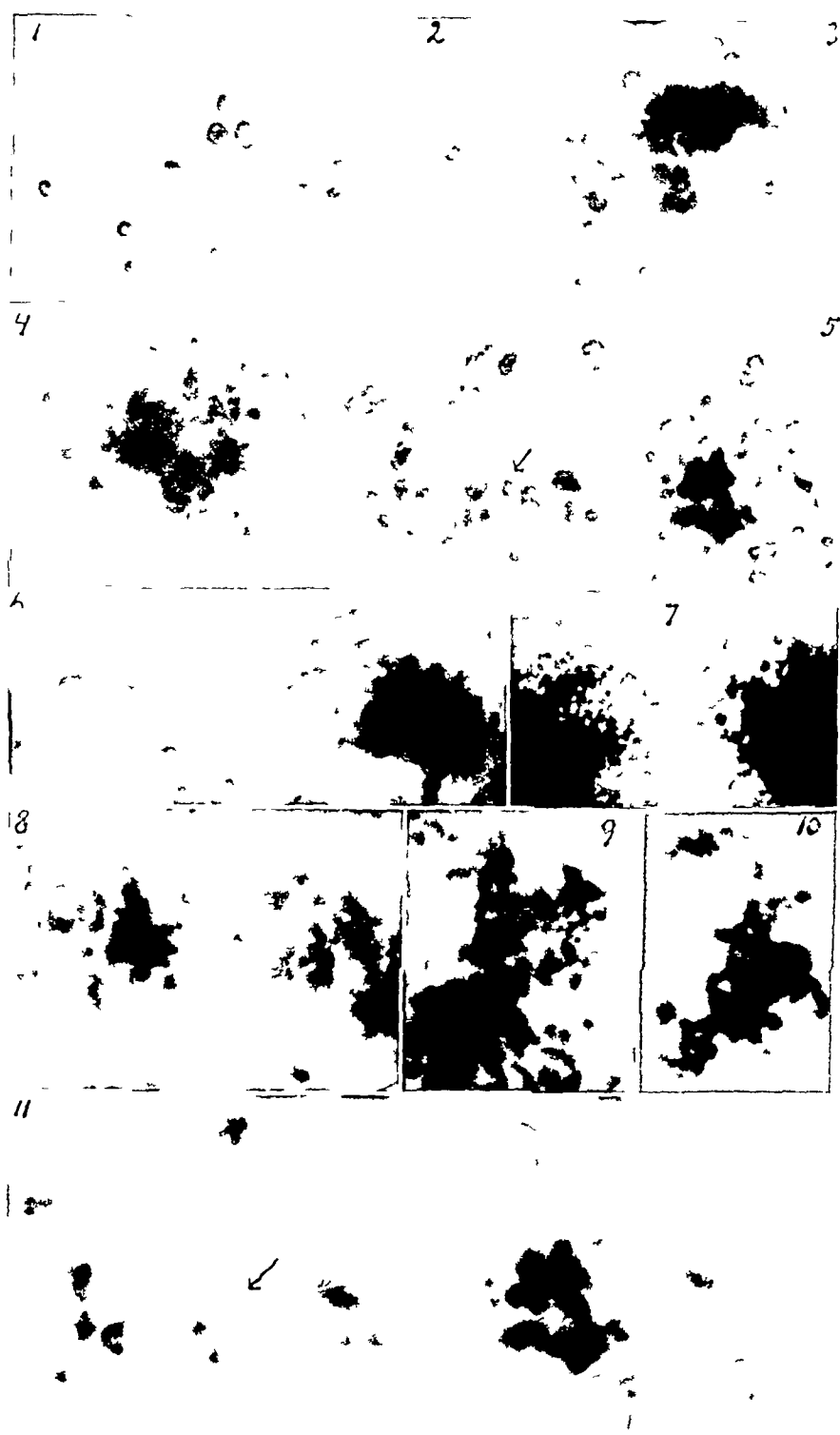
Fig 1
232

observe t e whole course of their development   The following studies are based on three strains of *Streptobacillus moniliformis* and the L_1 variants derived from them

The organisms in L_1 colonies appear in two main forms with intermediate transitional forms   The young colonies and the central mass of well-developed colonies consist of small forms   These are transformed at the surface and the periphery of the colonies by gradual swelling into large round forms similar in every respect to the large forms present in colonies of the pleuropneumonia group of organisms and of various bacteria

The shape of the small organisms appeared most clearly in preparations made from cultures on coagulated egg   The colonies grew on the surface of this medium without penetrating it   Impression preparations were made from the cultures following agar fixation   As mentioned above, only the surface of the colonies adheres to the glass, but in some preparations a few small forms were mixed with the large bodies usually present on the surface   The smallest organisms appeared to be distinctly bacillary   Photograph no 2 of figure 1 made from such a preparation shows a tiny bipolar-stained bacillus   In photograph no 1 of figure 1 three small organisms adhere to each other forming a small filament   Although they are darker stained than the organism in the following photograph, their shape is distinctly bacillary   A slightly swollen organism in the same photograph shows polar staining, which is more or less apparent in some of the larger forms

In wet stained agar preparations the appearance of the small organisms was similar   The youngest colonies consist of small bacillary forms sometime showing bipolar staining   Vigorously growing young colonies were obtained with the following procedure   The fresh medium was covered with an agar square cut from a 24-hour culture and incubated for 8 to 13 hours   The old agar square was discarded and the agar under it containing a fresh growth was studied   The organisms in photographs no 5 and 11 of figure 1 showed the bacillary form clearly and, in some cases, bipolar staining as well   It is hoped that their shape

FIGURE 1

No 1 Impression preparation from a young L_1 colony grown on coagulated egg   Fixation through the medium with Bouin's solution   Staining with methylene blue and azur × 3,000   Individual organisms are clearly visible, a short bacillary filament consisting of 3 bacilli, a bipolar-stained short bacillus, and consecutive stages in the development of large round forms   In one round form the polar staining remains visible

No 2 A bipolar-stained bacillus from the same preparation as no 1   × 3,000

Nos 3 and 4   The surface of small L_1 colonies photographed from wet stained agar preparation   × 3,000   In the lower part of no 3, a few bacillary forms are discernible besides moderately swollen forms   In no 4, the colony consists of small forms whose exact shape is not clearly visible

Nos 5, 6, and 11   Stained wet agar preparations   In no 6 (× 3,000), small bacillary forms and one round body are visible   In no 5 (× 3,000), many bacillary forms are visible, usually arranged in small clumps   One small bacillus marked with an arrow shows bipolar staining   No 11 is the same as no 5 enlarged to × 4,500   The shape of the organisms is only occasionally apparent, because they are parts of small clumps most of which, of necessity, are out of focus

No 7 Well developed colonies with moderate magnification   Stained dry agar preparation   × 500   The dense center consists of small forms embedded in the agar, the periphery consists of large round bodies situated on the surface of the agar

Nos 8, 9, and 10 Small L_1 colonies photographed from dried stained agar preparations × 3,000   The shape of individual organisms is not clearly visible, but it is apparent that their arrangement is similar to that of bacteria in bacterial colonies

and structure will remain visible in the reproductions   Although the fact that
the organisms are not in one plane and that they adhere in clumps makes it
difficult to obtain sufficiently clear photographs

Photographs no 8, 9, and 10 were made from preparations similar to the pre
ceding ones with the difference that the thin agar slices were dried on the cover
slips   Drying compresses the cultures vertically, and for this reason a large part
of the colony is seen in sharp focus   On the other hand, the individual organisms
are not so distinct as in wet preparations   The most important informations
obtained from the study of dry agar preparations is that the arrangement of the
organisms in the youngest colonies is similar to the arrangement of bacteria in
bacterial colonies, an observation indicating a similarity of growth and repro-
duction   The small bacillary forms in the photograph made from both wet and
dry preparations are about $\frac{2}{3}$ of a micron long and $\frac{1}{3}$ wide

The small forms in the L_1 colonies are transformed by gradual swelling into
large bodies   In photographs no 1 and 6 of figure 1 the consecutive stages of
this process are apparent   When the large bodies are fully developed, they some
times appear to be filled with small bacillary forms similar to those growing in
young colonies   These small forms are tightly packed in several layers, and their
actual forms can be seen only occasionally   They must be soft and fragile, be
cause even slight mechanical injury to the large body destroys them   The struc-
ture of the large bodies was most clearly seen in preparations stained with safra-
nine after fixation with Bouin's solution through the agar   Photographs 1 and 4
of figure 2 were made from such preparations

The development of the large bodies into L_1 colonies in transplants has been
previously described   They increase in size, their contours may become uneven,
and the small L_1 organisms grow out of them at one or at several points   The
large body does not germinate as a single organism, like a yeast cell, for example,
but it apparently contains numerous small organisms capable of growth   The
author agrees in this point with Klieneberger (1942), but he has seen no indication
of the process, postulated by Klieneberger, by which previously separate or-
ganisms develop a common membrane and form a large body   The gradual
swelling of the small forms into large bodies is apparent in the cultures

The development of L_1 has been followed on thin agar slices under a cover slip
and in stained agar preparations made from the cultures at short intervals   No
other viable organisms were discernible in the cultures except the small bacillary
forms, the large bodies, and the intermediary forms   The viable organisms are
stained intensely blue by methylene blue, and the cultures stop developing in
transplants when the blue staining disappears from the colonies   Autolysis pro
duced granules of various size which are stained pink both by methylene blue
and Giemsa solution   These granules never show multiplication in transplants
Similar granules are produced in autolyzed cultures of *Streptobacillus moniliformis*
and are regarded by Klieneberger as forms of L_1 (1942)   According to the ex
perience of the author these granules do not multiply and there is no reason to
believe that they represent the L_1 When a *Streptobacillus* culture which is not
grossly mixed with L_1 colonies is transplanted, the L_1 develops exclusively from
large bodies produced by swelling of the bacilli
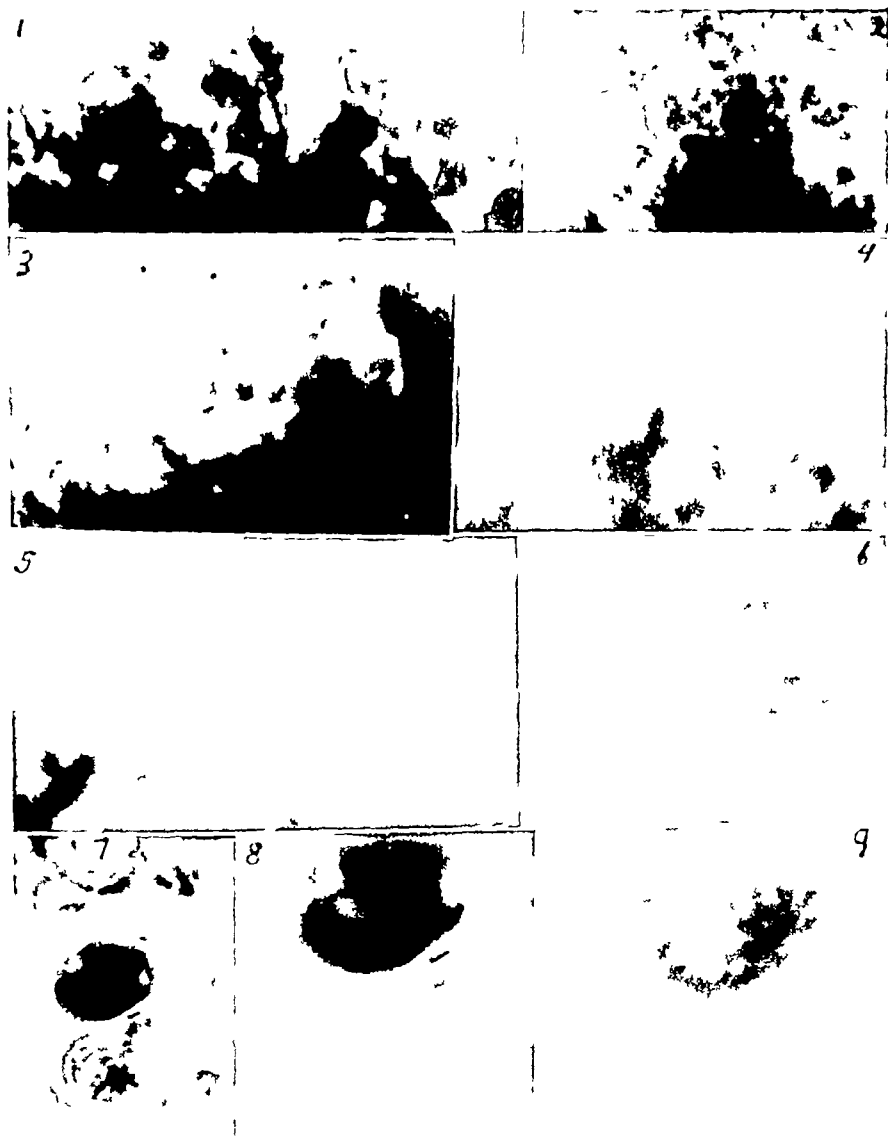
FIGURE 2

No 1 Safranine staining after agar fixation   × 3,000   Darkly stained small bacillary forms surrounded by halos are apparent in several large bodies
No 2 Large bodies filled with small bacillary forms in a culture of *Streptobacillus moniliformis*   Safranine after agar fixation   × 3,000
No 3 A large body in a culture of *Streptobacillus moniliformis* filled with round granules Methylene blue and azur after agar fixation   × 3,000
No 4 and 5 Enlargement of nos 1 and 2, respectively, to × 4,500
No 6 A large body in a *Streptobacillus* culture filled with bacilli of the usual shape Giemsa staining after agar fixation   × 3,000
No 7 Large body in a *Streptobacillus* culture filled with bacilli of the usual shape Giemsa staining after agar fixation   × 3,000
No 8 Same as no 7, enlarged to × 4,500
No 9 A large body similar to those in nos 6 and 7 stained with victoria blue   × 4,500

The development of L_1 in the cultures of *Streptobacillus moniliformis* from the the bacteria has been previously described (Dienes, 1942)   The bacilli swell first into large fusiform bodies and under appropriate conditions these develop into L_1 colonies   These large bodies are in appearance and physical properties similar to the large bodies of L_1 When they are fully developed, they are filled with similar soft bacillary forms and they develop into L_1 colonies in a similar way   Transformation of the usual bacilli into L_1 occurs during the development of the large body   Photographs no 2 and 4 of figure 2 show large bodies developing in cultures of *Streptobacillus moniliformis* filled with small bacillary forms   In photograph no 3 the small bodies developed into round forms, and it is clearly apparent that the large body contains many individual organisms

Although most of the large bodies develop as indicated above, in certain cultures some large bodies develop in a different way   These large bodies, a few hours after transplantation, appear to be filled with bacilli of the usual shape and develop into regular bacillary colonies   Then growth produces first a tiny dense round colony very different from the usual growth of *Streptobacillus*, after a few hours they lose this character and become similar to the other bacterial colonies The development of the large bodies into bacteria was described in a former paper (Dienes, 1943)   The illustrations in this paper were not successfully reproduced and are replaced here by better ones   Attention is again called to the fact that every strain of *Streptobacillus* and the L_1 isolated from it present marked individual properties   It is often impossible to observe in a given strain phenomena easily seen in others

DISCUSSION

The observations described give further support to the view that the morphology of the L_1 is bacterial   The small organisms in L_1 colonies are tiny, often bipolar-stained bacilli   Similar forms are visible inside the large bodies developing either in bacterial or L_1 cultures   These small bacillary forms share with the parent organism the tendency to swell to round forms   The L_1 is more pleomorphic and has a more pronounced tendency to autolysis than the parent organism, but it is essentially similar to the parent organism both in regard to form and to reproduction   The morphological differences between the L_1 and the parent organism were exaggerated by the use of inappropriate methods of observation   The differences between them are actually not more pronounced than those between a smooth and a very rough pneumococcus colony   It was mentioned above that large bodies found in the cultures of *Streptobacillus monil-iformis* can develop either into L_1 or into usual bacillary forms   This together with the observation that the L_1 for a certain period after isolation returns easily into the *Streptobacillus* indicates that the L_1 is apparently an intermediary link in the reproduction of the usual bacilli from the large bodies

All these observations are in agreement with the supposition that the usual bacillary forms and the L_1 are growth forms of the same organism   The only characteristic in the development of L_1 which was previously not noticed in bacterial variation is that the change into L_1 is preceded by a morphological change

of the parent organism, by swelling into large round forms   These processes are not exceptional in *Streptobacillus moniliformis*, but they are widely distributed in gram-negative bacilli (Dienes, 1942, 1946)   To all appearances these processes represent a complex reproductive process different from binary fission   The author has pointed out that the L₁ shows many similarities to the so-called "haploform" yeast of Winge (Dienes, 1946)

The morphology of the L₁ is similar in all essential characteristics to the pleuropneumonia group of organisms   Without knowing the origin of a culture after it has lost its ability to return into the *Streptobacillus*, it would be impossible to recognize its identity on the basis of morphology

SUMMARY

It is apparent in appropriate preparations that the small forms of the L₁ colonies are small, often bipolar-stained bacilli   They enlarge by gradual swelling into large forms in which the small bacillary forms are again reproduced   The large bodies produced by swelling of bacteria in cultures of *Streptobacillus moniliformis* contain similar small bacillary forms, and when they germinate they produce an L₁ colony   Sometimes the large bodies in the *Streptobacillus* culture are filled with bacilli of the usual shape and reproduce the usual bacillary colonies   The morphology of the L₁, like that of the whole pleuropneumonia group, is bacillary, and the swelling into large round forms and reproduction by these large forms is similar in nature to the analogous processes observed in other bacteria

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PRODUCTS OF ANAEROBIC GLYCEROL FERMENTATION BY STREPTOCOCCI FAECALIS

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The fermentation of oxidized or reduced substrates by homofermentative lactic acid bacteria must necessarily lead either to products other than lactic acid, or must require external hydrogen donors or acceptors   In a study of the fermentation of glycerol, a reduced substrate, by streptococci Gunsalus and Sherman (1942) noted among the enterococci two types of behavior, certain strains fermented glycerol readily with a limiting pH about 5, whereas others fermented the substrate slowly and reached a final pH of 5 5 to 6   The latter were found to require oxygen in order to utilize glycerol as an energy source   The glycerol metabolism of a strain of this type has been studied in some detail (Gunsalus and Umbreit, 1945)

In connection with the anaerobic fermentation of glycerol, Braak (1928) found with colon-aerogenes organisms that growth would cease before the glycerol was exhausted and would start again if more peptone or yeast extract was added   It seemed not unlikely that such a phenomena might also occur with lactic acid organisms

The present paper deals with a strain of *Streptococcus faecalis* that ferments glycerol readily, with good growth, under anaerobic conditions   With this strain, yeast extract in addition to glycerol is needed for anaerobic growth, whereas with glucose as substrate yeast extract is not required   The yeast extract can be replaced with fumaric acid if a sufficient level of riboflavin is present—the fermentation products being primarily lactic and succinic acids

METHODS

Culture   *Streptococcus faecalis*, strain 10C1, a typical enterococcus from the departmental culture collection, has been used throughout these studies   This strain, and others which ferment glycerol anaerobically, grows more abundantly in ordinary laboratory media than strains which ferment glycerol only aerobically   These strains also attack a wider range of substrates and yield a wider variety of products (Gunsalus and Campbell, 1944, Gunsalus and Niven, 1942)

Growth and media   The growth was measured turbidimetrically as described previously in papers from this laboratory (Gunsalus and Sherman, 1942)   Anaerobic conditions were obtained either by vaspar seals or the chromium-sulfuric-acid method as described by Mueller and Miller (1941)   The turbidity was measured at suitable intervals, and the final pH was determined at the end of the experiments, with a Beckman pH meter

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RESULTS

In order to estimate the amount of growth supported by glycerol as substrate, a comparison was made of the growth in the base medium, and in this medium with glycerol and with glucose as substrates   In these studies extra buffer was avoided in order that the influence of pH would not be further masked if slight fermentation of glycerol occurred

Influence of Yeast Extract and Oxygen

Since glycerol is more reduced than lactic acid, and since oxygen acts as an aerobic hydrogen acceptor in glycerol fermentation, it was considered possible that anaerobically some constituent of the medium might serve as a hydrogen acceptor   To test the effect of media constituents on growth and fermentation,

TABLE 1

Effect of yeast extract and oxygen upon growth on glycerol and glucose

Streptococcus faecalis 10Cl

Base Medium 1 per cent tryptone
Incubation 10 days, 37 C (anaerobic series in chromium sulfuric acid jar)

YEAST EXTRACT	AEROBIC GROWTH*			ANAEROBIC GROWTH		
	Base	Glycerol	Glucose	Base	Glycerol	Glucose
%						
0	14	21	125	12	14	125
0 2	19	52	170	19	45	160
0 5	28	70	200	30	60	170
1 0	47	115	210	44	74	190

* Turbidity 1 scale unit ≅ 6 μg bacterial N/10 ml

the yeast extract level was altered as shown in table 1   One per cent tryptone supports slight growth of *Streptococcus faecalis*, strain 10C1, and glycerol improves the growth a little, whereas the addition of glucose as an energy source results in abundant growth   Not only is the anaerobic growth with glycerol poor, but aerobic growth is also slight   Therefore the tryptone must be deficient in factors necessary for the hydrogen transport to oxygen, otherwise, aerobic glycerol fermentation should occur (Gunsalus and Sherman, 1942)   In the base medium yeast extract improves the growth slightly, and the further addition of glycerol provides moderate growth stimulation   The presence of oxygen affords some stimulation beyond that due to the presence of yeast extract, indicating that the quantity of hydrogen acceptor might be limiting   On the other hand, the final pH (5 0 with glycerol) may become limiting before maximum growth is attained   The growth, final pH, and titratable acidity for several variations in the medium are shown in table 2   Although very little acid was formed in the base medium the final pH with yeast extract alone fell to 6 0   In the presence of glycerol the limiting pH was reached in all media containing 0 5 per cent, or more, yeast extract regardless of the level of tryptone   The glycerol supported about on⌐

third the growth, and about one-third the acid production, afforded by glucose
Whether this is due to the difference in fermentation pattern, or more likely, to
the difference in growth afforded by the higher limiting pH with glycerol, cannot
be determined by these data   The growth with glycerol as substrate can be
about doubled by the addition of 0 5 per cent dipotassium phosphate, the limiting
pH is not always reached in this case   Glycerol will support growth and acid
production only when the yeast extract is added to the medium

Fractionation of Yeast Extract and Replacement with Fumaric Acid and Riboflavin

Initial attempts to fractionate the yeast extract, as by ether extraction, resulted
in two fractions neither of which was markedly active alone   However, on re-
combination they exhibited the full activity of yeast extract   Some fractions
caused lower pH without much stimulation of growth, whereas others stimulated

TABLE 2
Anaerobic glycerol fermentation
Streptococcus faecalis 10Cl
Incubation 8 days, 37 C in anaerobic jar

TRYPTONE	YEAST EXTRACT	GROWTH*			FINAL pH			ACID PRODUCTION		
		Base	Glycerol	Glucose	Base	Glycerol	Glucose	Base	Glycerol	Glucose
%	%							*ml N/10 acid/10 ml*		
1	0	16	19	88	6 9	6 4	3 9	1	0	3 6
1	0 5	18	56	90	6 6	5 2	4 0	0	1 1	3 9
1	1	22	58	110	6 7	5 1	4 1	0	1 6	4 6
0 5	1	18	44	74	6 6	5 0	4 1	4	1 0	4 8
0	1	10	24	53	6 0	4 9	4 0	4	4	4 4

* Turbidity 1 scale unit ≅ μg bacterial N

growth without as great a depression in pH, thus suggesting the possibility that
more than one substance was involved
    Therefore, several attempts were made to replace the yeast activity by known
compounds   As shown in table 3, with glycerol as substrate in a tryptone me-
dium, a small amount of yeast extract will stimulate growth only slightly but will
stimulate more rapid acid production—final pH 5 0   The acid production is also
stimulated by a mixture of accessory factors, or by riboflavin   In the presence
of traces of yeast extract, or accessory factors, fumaric acid will greatly stimulate
the growth   Although fumaric acid can replace the yeast extract, it does not
necessarily follow that the action of yeast extract is due to fumaric acid
    Presumably, the fumarate acts as a hydrogen acceptor and additional riboflavin
is needed for hydrogen transport   The data in table 4, with a synthetic medium,
show plainly the increased riboflavin requirement   With the riboflavin and fu-
marate, glycerol fermentation proceeds rapidly, 12-hour growths being recorded,
and appears not to require further factors beyond the requirement for growth in
glucose   It should be noted, however, that the growth with glycerol as substrate
still does not equal that with glucose

TABLE 3

Factors affecting glycerol fermentation

Incubation 2 days, 37 C Medium 1% tryptone

ADDITIONS	GROWTH*			pH		
	Base	Glycerol	Increase	Base	Glycerol	Decrease
None	18	30	12	7 2	6 0	1 2
Yeast extract, 0 05%	20	40	20	7 2	4 9	2 3
0 1%	24	42	18	7 1	4 9	2 2
1 0%	42	76	34	6 6	4 9	1 7
Accessory factors†	20	38	18	7 2	4 9	2 3
Riboflavin, 5 µg	20	32	12	7 2	5 1	2 1
Fumarate, 0 5%	16	36	20	7 2	5 5	1 7
Fumarate Yeast extract, 0 1%	20	110	90	7 2	5 3	1 9
Fumarate Yeast extract, 0 5%	27	180	153	7 2	5 3	1 9
Fumarate Accessory factors†	15	110	95	7 2	5 3	1 9
Fumarate Riboflavin, 1 µg	20	110	90	7 2	5 4	1 8

* Turbidity 1 scale unit $\cong$ 6 µg bacterial N per 10 ml

† Contains 2 5 µg thiamine, 5 µg each of riboflavin, pyridoxine, and *para* aminobenzoic acid, 20 µg pantothenic acid, 25 µg nicotinic acid, 1 mµg biotin, and 0 1 µg glutamine per tube

TABLE 4

Growth and acid production in synthetic medium

Per tube 10 ml base medium of Bellamy and Gunsalus (1945) omitting riboflavin and glucose

Incubation 12 hours, 37 C

RIBOFLAVIN	0 5 GLUCOSE		0 5% GLYCEROL		0 5% GLYCEROL 0 5% FUMARATE	
µg/tube	Growth	pH	Growth	pH	Growth	pH
0	10	7 3	4	7 3	4	7 2
0 1	100	5 6			31	7 0
0 2	100	5 1	20	7 1	46	6 6
0 4	100	5 1	20	7 1	50	6 4
1 0	86	5 1	18	7 3	65	6 1
10 0	90	5 1	20	7 3	56	6 2

Fermentation Products from Glycerol and Glycerol-Fumarate

Fermentation balances with *Streptococci faecalis* demonstrated that approximately 95 per cent of the glucose fermented appears as lactic acid (Smith and Sherman, 1942) The fermentation pattern can, however, be altered by alkaline reaction (Gunsalus and Niven, 1942) or with oxidized substrate (Gunsalus and

Campbell, 1944)   The fermentation products, with an oxidized substrate such as citric acid, are largely acetic and formic acids and carbon dioxide, with only a trace of lactic acid, thus indicating that while this organism is homofermentative on a balanced substrate, other fermentative potentialities are present   In contrast to the change in products with oxidized substrate, glycerol, a reduced substrate, yields mainly lactic acid in a tryptone yeast-extract base medium (table 5) The two extra hydrogens which arise from glycerol are largely unaccounted for

A more marked fermentation, accompanied by increased growth, occurs in the presence of glycerol and fumarate   In this case the products are mainly lactic and succinic acids (table 5)   In buffered media, especially in the presence of calcium carbonate and an excess of riboflavin, the fermentation can be further altered so that the quantity of fumarate reduced to succinate is greater than the

TABLE 5

Products of glycerol and of glycerol fumarate fermentation

Base medium 1% tryptone, 0 2% yeast extract, 0 2% K_2HPO_4
Incubation 3 days, 37 C Substrates added aseptically

PRODUCTS	1% GLYCEROL	1% GLYCEROL 1% FUMARATE
	$mM/100\ ml$	$mM/100\ ml$
Lactic acid	1 9	3 04
Succinic acid	0 46	3 13

Base medium as above 1% $CaCO_3$

SUBSTRATES FERMENTED		PRODUCTS FORMED	
	$mM/100\ ml$		$mM/100\ ml$
Glycerol	7 1	Lactate	3 2
		Acetate	2 3
Fumarate	7 5	Succinate	7 5

lactic acid formed (table 5)   In this case more oxidized products, acetic acid and carbon dioxide, account for the rest of the glycerol fermented   It is not surprising that under conditions in which fumarate is a good hydrogen acceptor the fermentation is altered in the direction of oxidized products, since it has previously been shown that this organism contains a very active Kreb's dismutation for the formation of acetic and lactic acids and carbon dioxide (Miller, 1942), as well as a system for the conversion of pyruvate to formic and acetic acids (Gunsalus and Campbell, 1944)   This would indicate that hydrogen from triose-phosphate, as well as from the glycerol (phosphate), can be transferred to fumarate

DISCUSSION

The anaerobic fermentation of glycerol by streptococci is dependent upon the presence of external hydrogen acceptors, the main pathway of fermentation and energy liberation proceeding by the usual lactic acid pathway   This is contrary

to the results with oxidized substrates in which a series of oxidized products are formed with energy liberation during fermentation   However, the result is similar to that found by Braak (1928) in the colon-aerogenes group   Thus it appears that these organisms are not able to carry out a more reduced type of fermentation than the lactic scheme

The nature of the hydrogen acceptor of yeast extract that is available to this lactic organism is unknown and might bear investigation   Also, while the mechanism of the fermentation scheme with fumaric acid seems obvious, the nature of the enzymes should be determined, especially since a fumarate reductase (succinoxidase?) system in lactic acid bacteria appears not to have been previously reported

Taxonomic considerations could call for a review of the relationship of the aerobic and anaerobic glycerol fermentation types of enterococci to *Streptococcus foecium* and *Streptococcus glycerinaceous*, respectively, of Orla-Jensen (1919)

SUMMARY

Glycerol fermentation by streptococci has been found to occur only in the presence of external hydrogen acceptors, the main reaction being
$$\text{glycerol} \rightarrow \text{lactic acid} + 2H$$
Some strains, as described previously, can use only oxygen as a hydrogen acceptor, the other product being H_2O_2
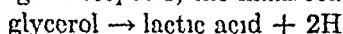
Other strains, as reported in this study, can use an unidentified substrate in yeast extract as hydrogenacceptor   This can be replaced by fumaric acid, in which case the main reaction becomes
$$\text{glycerol} + \text{fumaric acid} \rightarrow \text{lactic acid} + \text{succinic acid}$$
This reaction requires a higher riboflavin level than is necessary for glucose fermentation, very probably for hydrogen transport to fumaric acid   With an excess of fumarate, oxidized products are formed
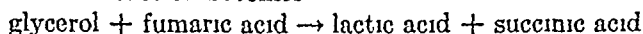
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THE "REVERSAL," NEUTRALIZATION, AND SELECTIVITY OF GERMICIDAL CATIONIC DETERGENTS[1]

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The reversal of the inhibitory action of antiseptics by substances showing a specific affinity for the inhibiting agent has been reported for many compounds, the most striking effect probably being the reversal of the action of mercurials by H_2S, glutathione, sodium thioglycolate, etc Valko and DuBois (1944) have reported that the germicidal action of cationic detergents against both gram-positive and gram-negative bacteria can be reversed by the anionic detergent duponol PC (sodium lauryl sulfate)

In the present work we have studied the ability of anionic detergents to reverse or neutralize the action of cationic detergents We have found that anionic detergents did not *reverse* the germicidal action of cationic detergents against either gram-positive or gram-negative bacteria If the cationic detergent inactivated the bacteria, the addition of an anionic detergent did not result in any reactivation However, when the anionic detergent was added *before* all the bacteria in a given inoculum were inactivated by the cation, the anionic detergent could *neutralize* the action of the cation against *gram-negative* bacteria and prevent any further germicidal action on the surviving bacteria Against *gram-positive* bacteria the bacteriostatic action of the cationic detergent was not neutralized by an anionic detergent even though the anion was added before the cation The failure to neutralize the action of a cationic detergent against gram-positive bacteria was found to be related to the high degree of selectivity shown by the compound for gram-positive bacteria

EXPERIMENTAL

Choice of neutralizing agent The anionic detergent duponol PC (sodium lauryl sulfate) was used as a neutralizing agent by Valko and DuBois in their studies on the reversibility of the bactericidal action of the cationic detergents However, in extract broth, pH 7 2, sodium lauryl sulfate was itself inhibitory against *Staphylococcus albus* in dilutions as high as 1 20,000 We therefore examined a group of anionic detergents in an effort to find an agent that was not bacteriostatic against the relatively susceptible gram-positive bacteria but was effective as a neutralizing agent against the cationic detergents We determined the bacteriostatic action of 12 anionic detergents[2] by seeding various

dilutions of the anionic detergents in extract broth, pH 7 2, with 0 1 ml of a 20- to 24-hour broth culture of *S albus* and determining the degree of inhibition after 24 hours' incubation at 37 C

The neutralizing action of the compounds against the cationic detergent zephiran was determined by adding various dilutions of the anions to equal volumes of 1 5,000 zephiran broth   The solutions were seeded with 0 1 ml of a 20- to 24-hour broth culture of *Escherichia coli*, and the lowest concentration of anionic detergent inhibiting the action of zephiran was determined   *E coli* was used as the test strain in the neutralization tests since anionic detergents have little activity against gram-negative bacteria at pH 7 2

The anionic detergents were found to vary markedly in their ability to neutralize the action of cationic detergents, and there was in general a correlation between the degree of inhibition of *S albus* and the extent of the neutralization of zephiran against *E coli*   No compound was found which was completely noninhibitory against *S albus* and still effective in neutralizing the action of zephiran against *E coli*   Sodium decyl sulfate was found to be the best neutralizing agent and the only compound which combined a low bacteriostatic activity with a high degree of neutralizing action against *E coli*   It was only slightly inhibitory against *S albus* in a 1 5,000 broth dilution, was not inhibitory at a 1 10,000 dilution, and was completely inactive against the gram-negative bacilli in a 1 5,000 broth dilution, pH 7 2   The addition of an equivalent volume of a 1 5,000 dilution of the compound permitted the growth of *E coli* in a 1 5,000 broth dilution of zephiran   Sodium decyl sulfate was also found to neutralize effectively the action of the cationic detergents ceepryn, phemerol, and emulsol 607 against *E coli*

Failure to obtain reversal of the germicidal action of the cationic detergents   In our studies on the ability of the anions to reverse the action of cationic detergents, we first repeated the procedure used by Valko and DuBois   Three anionic detergents were used as neutralizing agents   duponol C, a commercial grade of alkyl sulfates, primarily sodium lauryl sulfate, sodium lauryl sulfate, and sodium decyl sulfate   Our test procedure followed that of Valko and DuBois though, in addition to broth subcultures, plate counts were used to determine quantitatively the degree of reversal   To varying dilutions of the cationic detergents in distilled water, 0 5 ml of a 20- to 24-hour broth culture of the test bacteria were added   After 5 minutes at room temperature (several tests were run at 37 C with similar results), 4 loops were subcultured into extract broth and a 1-ml sample was removed and diluted in saline, counts were determined in extract agar   Immediately after the removal of the sample for plating 0 5 ml of the test concentration of the anionic detergent were added to the solution of cationic detergents   Five or 10 minutes (several tests were also done at 30 minutes) after the addition of the anionic detergents, broth subcultures were again made and a 1-ml sample was removed, diluted in saline, and plated out to determine the degree of reversal effected by the anionic detergent   Four test strains were used, *Staphylococcus aureus*, *Escherichia coli*, *Shigella paradysenteriae* (Flexner), and *Salmonella schottmuelleri*   A total of 30 assays were done with

the cationic detergents zephiran and phemerol   Though the dilutions of the cationic detergents and the anionic detergents varied from 1 3,000 to 1 20,000 and the ratio of cation to anion varied from 1 1 to 1 6, in no case was any evidence of reversal obtained in the germicidal range of the cationic detergents As determined by plate counts, there was never any increase in the number of viable bacteria after the addition of the neutralizing agent   Broth subcultures from the germicidal concentrations of zephiran and phemerol were always negative, and all subcultures remained negative after the addition of the neutralizing agent

Neutralization of the action of cationic detergents against gram-negative bacteria Though no reversal was obtained with the germicidal concentrations of zephiran or phemerol, it was thought that reversal might be effected with the more dilute concentrations of zephiran in the bacteriostatic range   With sodium decyl sulfate as the neutralizing agent, the following test procedure was used   For each test a duplicate series of broth tubes containing dilutions of zephiran ranging from 1 1,000 to 1 3,000,000 were seeded with 0 1 ml of a 20- to 24-hour broth culture of the test bacteria   After the bacteria were in contact with zephiran for 15 minutes at room temperature, equal volumes of sodium decyl sulfate in a broth dilution of 1 5,000 (1 15,000 and 1 25,000 sodium decyl sulfate were also used against the gram-positive bacteria) were added to one series of tubes, and equal volumes of broth were added to the control zephiran tubes   The cultures were incubated at 37 C for 24 hours, and the titers obtained with the zephiran alone were compared with the titers obtained with the zephiran and sodium decyl sulfate   A total of 17 gram-negative and gram-positive bacteria were studied   As shown in table 1, sodium decyl sulfate differed markedly in its zephiran-neutralizing action when tested against the gram-positive bacteria (and meningococcus) and the gram-negative bacteria   There was only a slight degree of neutralization of zephiran action against 3 of the 10 gram-positive bacteria, but effective neutralization was obtained with all of the gram-negative bacteria

In order to determine whether growth in broth tubes to which sodium decyl sulfate was added was an actual reversal of zephiran action on the gram-negative bacteria or merely an interruption of the continued action of zephiran (neutralization) the foregoing test procedure was modified as shown in table 2   The data reveal that the growth obtained after the addition of sodium decyl sulfate was not a reversal phenomenon, as indicated by the absence of any significant increase in the bacterial count   Sodium decyl sulfate merely interrupted the continued action of the zephiran, upon further incubation the viable gram-negative bacteria present at 15 minutes in zephiran alone were inhibited, whereas the addition of the sodium decyl sulfate neutralized the zephiran and permitted the surviving bacteria to grow out

Failure to obtain neutralization of the action of zephiran against the gram-positive bacteria Though viable gram-positive bacteria were present in the high zephiran dilutions at the time of the addition of the sodium decyl sulfate, no significant degree of neutralization was obtained (table 1)   Inhibition of growth by the

sodium decyl sulfate was not a factor in the failure to obtain neutralization since a 1 30,000 broth dilution (equivalent to the 1 15,000 sodium decyl sulfate added to an equal volume of zephiran) permitted the growth of an inoculum of 10 to 100 bacteria from a *S aureus* broth culture   As shown in table 3, neither sodium decyl sulfate nor sodium lauryl sulfate was able to neutralize the *bacterio*

TABLE 1

Neutralization of the bacteriostatic action of zephiran broth by sodium decyl sulfate

GRAM POSITIVE BACTERIA	ZEPHIRAN BROTH*		ZEPHIRAN BROTH PLUS 1 5 000 SODIUM DECYL SULFATE†	
	Inhibited by	Growth in	Inhibited by	Growth in
Staphylococcus aureus 5A	1 1,000‡	1 2,000	1 800	1 1,000
Streptococcus pyogenes	1 800	1 1,000	1 600	1 800
Bacillus subtilis	1 1,000	1 2,000	1 800	1 1,000
Staphylococcus aureus 4A	1 1,000	1 2,000	1 1,000	1 2,000
Staphylococcus albus	1 800	1 1,000	1 800	1 1,000
Bacillus mycoides	1 800	1 1,000	1 800	1 1,000
Sarcina lutea	1 2,000	1 3,000	1 2,000	1 3,000
Gaffkya tetragena	1 2,000	1 3,000	1 2,000	1 3,000
Diplococcus pneumoniae	1 200	1 400	1 200	1 400
Neisseria intracellularis	1 400	1 500	1 400	1 500
GRAM NEGATIVE BACTERIA				
Eberthella typhosa	1 40	1 80	1 5	1 10
Shigella paradysenteriae (Flexner)	1 200	1 400	1 20	1 40
Escherichia coli	1 100	1 200	1 5	1 10
Proteus vulgaris	1 20	1 40	1 1	1 5
Salmonella schottmuelleri	1 100	1 200	1 10	1 20
Salmonella paratyphi	1 10	1 20	1 1	1 5
Pseudomonas aeruginosa	1 1	1 3		1 1

* *Neisseria intracellularis, Streptococcus pyogenes*, and *Diplococcus pneumoniae* were grown in Difco phenol red glucose broth, pH 7 2   The activity of zephiran in this medium was less than in the extract broth, pH 7 2, used in all other assays

† A 1 15,000 sodium decyl sulfate broth was also used as a neutralizing agent against all of the gram-positive bacteria and *Neisseria intracellularis*   A 1 25,000 sodium decyl sulfate broth was also used against *Streptococcus pyogenes, Diplococcus pneumoniae*, and *Neisseria intracellularis*   Titers were similar at all concentrations of the neutralizing agents   All readings were taken after 24 hours' incubation at 37 C

‡ Indicated dilutions $\times 10^{-3}$

static action of zephiran against gram-positive bacteria when added *before* the bacteria   The addition of sodium decyl sulfate to the zephiran before the addition of the *gram-negative* bacteria did result in effective neutralization

Though the anionic detergent did neutralize the immediate *germicidal* action of zephiran against gram-positive bacteria, the bacteria were not capable of growing   For example, when 1 10,000 or 1 20,000 sodium lauryl sulfate broth was added to equal volumes of 1 10,000 or 1 20,000 zephiran broth and then seeded with *S aureus*, the rapid germicidal action of zephiran was neutralized, but after 24 hours' incubation the viable bacteria failed to grow and plate counts

TABLE 2
Neutralization of action of zephiran by sodium decyl sulfate
(Escherichia coli)

	ZEPHIRAN BROTH DILUTIONS			
	1 10,000	1 20,000	1 40,000	Broth Contro
A 15 min in zephiran broth Bacteria per ml	7×10^2	11×10^4	$3\,2 \times 10^6$	$1\,5 \times 10^8$
Growth in 24 hr	0	0	0	++++
B 15 min in zephiran broth 1 4,000 sodium decyl sulfate broth added After 15 min bacteria per ml	17×10^2	$7\,8 \times 10^4$	$5\,4 \times 10^6$	
Growth in 24 hr	+++	++++	++++	++++

The titer of zephiran in this assay was lower than it was in previous assays

Similar results were obtained in several assays with *Shigella paradysenteriae (Flexner)* and *Salmonella paratyphi*

TABLE 3
Neutralization of action of zephiran by sodium decyl sulfate
(Staphylococcus aureus strain 4A)

	ZEPHIRAN BROTH DILUTIONS								
	1 25*	1 50	1 100	1 200	1 400	1 600	1 800	1 1 000	Control
Zephiran broth Growth in 24 hr	0	0	0	0	0	0	0	+++	++++
Zephiran broth plus 1 15,000 sodium decyl sulfate Growth in 24 hr	0	0	0	0	0	0	0	+++	++++
Zephiran broth plus 1 50,000 sodium decyl sulfate Growth in 24 hr	0	0	0	0	0	0	0	+++	++++

(Eberthella typhosa)

	1 5*	1 10	1 20	1 40	1 80	Control
Zephiran broth Growth in 24 hr	0	0	0	0	+++	++++
Zephiran broth plus 1 10,000 sodium decyl sulfate Growth in 24 hr	+++	+++	+++	+++	++++	++++

Equal volumes of zephiran broth and sodium decyl sulfate broth combined before seeding with 1 loop of a 20-hour culture of *S aureus* or *E typhosa* Similar results were obtained with *S aureus* when 1 25,000 and 1 50,000 sodium lauryl sulfate were used as the neutralizing agent

* Indicated dilution $\times 10^{-3}$

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made after 6 and 24 hours' incubation showed a gradual reduction in the number of viable bacteria

Selectivity of the action of cationic detergents   On the basis of *germicidal* activity and the inhibition of bacterial respiration (Baker, Harrison, and Miller, 1941) the cationic detergents have been found to be relatively nonselective in their activity against gram-positive and gram-negative bacteria, showing only a slightly greater activity against gram-positive bacteria than against gram negative bacteria   In the present studies on the bacteriostatic activity of zephiran, however, the compound, as shown in table 1, has, after neutralization, several hundredfold greater activity against the gram-positive bacteria (and *Neisseria intracellularis*) than against the gram-negative bacteria, a selectivity quite similar to inhibitors such as penicillin or gentian violet

<div align="center">DISCUSSION</div>

Our results have shown that anionic detergents are not capable of reversing *the action of cationic detergents in a manner analogous to the reversal* obtained with H₂S acting on HgCl₂   Hotchkiss (1946) has reported that relatively high concentrations of cationic detergents damage the bacterial cell with a subsequent release of the intracellular constituents into the surrounding medium   HgCl₂ did not effect this destruction of the cell   Under such conditions one would not expect any reversal of the action of high concentrations of zephiran, and no reversal was obtained in the present work

Our results on the selectivity of action of zephiran on gram-positive bacteria indicate that it has essentially the same bacterial spectrum as an agent such as penicillin   The recognition of the selectivity of action of penicillin and the classification of zephiran as a relatively nonselective inhibitor is probably due to the fact that we routinely test our chemotherapeutic agents by means of bacteriostatic tests, whereas germicides are ordinarily assayed by means of the phenol coefficient test   The high concentrations required for the bactericidal action of the compound obscured the selective action in the bacteriostatic range

The high bacteriostatic activity of zephiran against the gram-positive bacteria and the failure of anionic detergents to neutralize this action indicates a specificity of action not revealed in the studies on the inhibition of bacterial respiration (Baker, Harrison, and Miller, 1941)   Gale and Taylor (1946) have found that the assimilation and concentration of glutamic acid is restricted to the gram positive bacteria and this mechanism is specifically inhibited by penicillin   In view of the similarities in the susceptibility of gram-positive and gram-negative bacteria to penicillin and zephiran, it is possible that the relatively selective gram-positive bacteriostatic activity of zephiran may be related to a similar mechanism

<div align="center">SUMMARY</div>

The activity of the cationic detergents zephiran and phemerol against gram-positive and gram-negative bacteria could not be reversed by the anionic detergents sodium decyl sulfate or sodium lauryl sulfate

Anionic detergents neutralize the bacteriostatic action of zephiran against gram-negative bacteria but do not neutralize the bacteriostatic action of zephiran against gram-positive bacteria

The cationic detergent zephiran in its bacteriostatic range has a high degree selectivity and possesses several hundredfold greater activity against gram-positive bacteria than against gram-negative bacteria

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TUBERCULOSTATIC AND TUBERCULOCIDAL PROPERTIES OF STREPTOMYCIN[1,2]

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Antibiotics are frequently looked upon as primarily bacteriostatic agents, in spite of the fact that their bactericidal properties have been definitely established   The reasons for such assumptions are dependent largely upon the following characteristic properties of antibiotic substances 1 Compared to chemical antiseptics, antibiotics exert a much slower bactericidal effect upon sensitive organisms   2 The bactericidal action of an antibiotic depends upon a number of factors, as, for example, the age of the bacterial culture, penicillin is largely bacteriostatic against old cultures but it is bactericidal as well for young, rapidly growing cultures of bacteria   3 Frequently a much larger concentration of the antibiotic is required to kill the bacterial cells than is necessary to inhibit their growth   4 When a few viable cells are left in the culture to which the antibiotic has been added, especially when those cells are more resistant to the action of a given concentration of the antibiotic, they begin to develop rapidly, giving rise to a more resistant culture, the impression may thus be produced that the antibiotic has only a limited bactericidal effect

In a comparative study of the bactericidal action of a number of antibiotics (Waksman and Reilly, 1944), the conclusion was reached that those agents which are characterized by a high bacteriostatic action against a certain organism are also strongly bactericidal   This action depends upon both the nature of the organism and that of the antibiotic

The use of streptomycin in the chemotherapy of tuberculosis has recently focused particular attention on the problem of the bacteriostatic vs bactericidal action of this antibiotic   The fact that tubercular infection in experimental animals is not rapidly eliminated by treatment with streptomycin, an antibiotic which possesses marked antituberculosis properties (Schatz and Waksman, 1944), gave the impression that this antibiotic acts largely as a bacteriostatic rather than as a bactericidal agent (Hinshaw and Feldman, 1945)   This conclusion was based primarily on the fact that streptomycin does not bring about complete sterilization of the animal body   Upon cessation of streptomycin therapy, the few remaining cells which in the meantime may have developed resistance may begin to multiply again   A state of reinfection may thus be brought about, giving the impression that the antibiotic did not exert any bactericidal action at all

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It has been amply demonstrated (Waksman, 1947), however, that various anti_ biotics exert a marked bactericidal effect upon the gram-positive and gram-nega tive bacteria   Although the early demonstration (Schatz and Waksman, 1944) of the bactericidal action of streptomycin may be considered as inconclusive (Middlebrook and Yegian, 1946), lack of suitable methods for the enumeration of tubercle bacteria prevented at that time the presentation of results in more accurate form, although there was no doubt that the antituberculosis effect of the antibiotic was also markedly bactericidal   By the use of a rather crude tech nique it was demonstrated that when 200 to 300 μg per ml of streptomycin were allowed to act upon clumps of the human strain of the tubercle organism (H37) for several days at 37 C, there was a striking reduction in the number of viable cells

Streptothricin, an antibiotic closely related to streptomycin, was also found (Woodruff and Foster, 1944) to exert a tuberculocidal action, three units of streptothricin per milliliter of culture being required to kill all the cells of the nonpathogenic strain of *Mycobacterium tuberculosis* no 607 in 14 days, a bacterio static effect was brought about by only 0 3 to 1 0 unit

The bactericidal action of streptomycin upon the tubercle organism in experi mental infections as well as in clinical tuberculosis has also been established This hardly justifies the previous statement that streptomycin is largely tuber culostatic and not tuberculocidal   This was well expressed recently by Dr Feldman (1947) "Evidence obtained from clinical sources indicates quite defi nitely a marked diminution in the number of tubercle bacilli that can be demon strated by cultural means from such materials as bronchial secretions, gastric washings and urine after the patient has been under treatment with streptomycin for some time "

Recently, suitable media have been developed (Dubos and Davis, 1946) for obtaining diffuse growth of *M tuberculosis* throughout the culture   This per mitted the use of accurate turbidimetric methods (Smith, 1947) for the quanti tative estimation of the growth of the organism and for the evaluation of the effect of antibiotics upon the course of its multiplication   When agar is added to such media, the exact number of living cells of *M tuberculosis* in a given suspension can be determined, thus making it possible to measure accurately both the tuberculostatic and the tuberculocidal action of an antibiotic, and to interpret more closely the relative significance and the interrelations of these two phenomena in the survival of this organism in an artificial culture or in the body of the host

EXPERIMENTAL

Organisms and methods   The following investigations were undertaken for the purpose of clarifying the bacteriostatic vs the bactericidal effects of streptomycin upon certain acid-fast bacteria   For this purpose, various saprophytic and pathogenic organisms were used   Most of the cultures, with the exception of the two human pathogens, were obtained from the American Type *Culture* Collection

These *Mycobacterium* cultures were *M phlei*, the so-called timothy hay bacillus, *M avium*, *M tuberculosis* var *hominis* no 607, nonpathogenic strain, *M tuberculosis* var *hominis* H37Rv, pathogenic strain, *M tuberculosis* var *hominis* H37RvR, streptomycin-resistant pathogenic strain obtained from Dr Youmans

The method of growing the organisms for bacteriostatic tests has been described elsewhere (Smith, 1947)   For bactericidal studies, the cultures were plated out on suitable agar media, incubated for varying periods of time at 37 C, and all the colonies counted

Bacteriostatic and bactericidal action of streptomycin   The results of several experiments are reported in order to demonstrate the relationship between the antituberculosis effect of streptomycin, its concentration, and the length of the incubation period   In the first two experiments, the nonpathogenic strain of

TABLE 1

Bacteriostatic effect of streptomycin on Mycobacterium tuberculosis no 607

INCUBATION	STREPTOMYCIN, MICROGRAMS PER MILLILITER						
	0 0	0 1	0 2	0 3	0 5	1 0	2 0
	Turbidimetric readings (in logs)						
hours							
6	0	0	0	0	0	0	0
12	3	3	0	0	0	0	0
24	18	13	3	0	0	0	0
48	45	31	7	0	0	0	0
72	53	4S	10	0	3	0	0
240	155	15S	112	0	4	0	0

M tuberculosis was used   When a short incubation period of 12 hours is used, 0 2 μg per ml is sufficient to inhibit the growth of the organism   When incubation of the cultures is continued for a longer period, however, 0 3 μg per ml becomes the inhibiting concentration   In one set of cultures a limited amount of growth was obtained upon prolonged incubation, even in the presence of 0 5 μg per ml of the antibiotic (table 1)

The bactericidal effect of streptomycin upon the nonpathogenic strain is shown in table 2   Here as well, the incubation period was found to be of great importance   Although a certain bactericidal action was obtained even with 0 3 μg per ml of the antibiotic, especially after a longer incubation period, it took more than 2 μg per ml to kill all the cells in 6 hours, a similar effect was exerted by 1 μg per ml when incubation was continued 24 hours   With lower concentrations of streptomycin, it took more than 24 hours to destroy all the cells, as detected by the plate method   The smallest concentration of streptomycin, namely, 0 2 μg per ml, exerted chiefly a bacteriostatic effect

The pathogenic strain of *M tuberculosis* H37Rv grows more slowly   More streptomycin was required to inhibit its growth than was the case with the non-

pathogenic strain it took 2 µg per ml of streptomycin to bring about complete inhibition, and 1 µg per ml, for partial inhibition (table 3)

In view of the fact that it is generally recognized that the size of the inoculum has a decided influence upon the antibiotic effect of a given agent, a study was made of the significance of the inoculum size upon the tuberculostatic vs tuber

TABLE 2

The bactericidal activity of streptomycin on Mycobacterium tuberculosis no 607

INCUBATION	STREPTOMYCIN, MICROGRAMS PER MILLILITER					
	0 0	0 2	0 3	0 5	1 0	2 0
	Numbers of viable cells per milliliter*					
hours						
6	283,000	229,000	83,800	26,700	1,120	40
12	1,800,000	427,000		3,000	490	0
24	18,900,000	1,660,000	200	220	0	0
48	12,650,000	1,770,000		700	0	0
72	16,500,000	3,944,000	0	2,500	0	0
240	9,400,000	6,330,000	0†	7,500	0	0

* Number at start, 216,000
† In a duplicate series, 20,900,000 cells per ml were found after 240 hours' incubation in the presence of 0 3 µg per ml of streptomycin

TABLE 3

The bacteriostatic action of streptomycin on Mycobacterium tuberculosis
var hominis strain H37Rv

INCUBATION	STREPTOMYCIN, MICROGRAMS PER MILLILITER				
	0 0	0 6	1 0	2 0	4 0
	Turbidimetric readings (in logs)				
days					
1	7	1	0	2	2
4	42	16	10	6	4
7	83	37	12	5	4
11	130	68	12	0	0
14	186	92	13	0	0

culocidal properties of streptomycin   For this purpose, *M avium* was used The results (table 4) show that there is a definite direct correlation between the size of inoculum and the amount of streptomycin required to inhibit the growth of the organism   More of the antibiotic was required to inhibit *M avium* when 0 1 mg per ml of inoculum was used than with only 0 001 mg per ml or less of the cell material   The same was true for the bactericidal action of streptomycin   it took four times as much streptomycin to inhibit the growth of, or to kill, all the cells capable of developing on the plate when 0 1 mg per ml of the inoculum was used than when 0 00001 mg per ml was used

Development of resistant cells in cultures containing varying amounts of streptomycin is shown by the results of a typical experiment (table 5)   As

TABLE 4

Influence of inoculum on the bacteriostatic and bactericidal action of streptomycin on Mycobacterium avium

INCUBATION	CONCENTRATION OF CELL MATERIAL IN MG/ML			
	0 1	0 01	0 001	0 00001
	μg/ml of streptomycin required for bacteriostasis			
days				
1	0 4	0 4	0 05	0 0
3	0 6	0 4	0 4	0 2
7	0 8	0 4	0 4	0 2
	μg/ml of streptomycin required for bactericidal action*			
1	>1 0	>1 0	1 0	0 6
3	0 8	0 4	0 4	0 4
7	0 8	0 4	0 4	0 2

* Amounts required to give complete destruction of all cells

TABLE 5

Influence of age of culture of Mycobacterium tuberculosis no 607 upon the development of cells resistant to different concentrations of streptomycin

AGE OF CULTURE	NUMBER OF CELLS (THOUSANDS/ML) IN MEDIA CONTAINING DIFFERENT CONCENTRATIONS OF STREPTOMYCIN (μG/ML)			
	0 0	0 3	0 5	0 7
hours				
0	30	10	0	0
2	32	10	0 2	0 01
4	33	0	0	0
6	35	17	0	0
22	3,600	1,550	6 6	0
30	13,560	5,050	3 6	0 4
42	21,800	2,500	70 0	0 6
48	40,450	11,400	10 0	0 3
66	75,100	2,600	800 0	1 4
89	130,000	19,500	2 1	3 7
96	143,000	7,200	2 2	2 5
114	425,000	12,680	5 8	5 4
168	650,000	25,000	38 3	5 8
235	503,500	1,300	1 6	7 9
336	1,130,000	28,200	15 0	6 5
504	561,000	63,500	570 0	40 7

cultures developed, there was actually a decrease rather than an increase in the proportion of resistant cells   The percentage of cells resistant to 0 3 μg per ml of streptomycin decreased from 30 per cent at the start to 15 per cent or less

after 48 hours' incubation   In a growing culture the percentage of resistant cells to larger amounts of streptomycin was even less, the sensitive cells not being eliminated at the expense of the resistant cells

The combined effect of two antibiotics upon M tuberculosis   Simultaneously with the extensive application of streptomycin in the treatment of clinical tuber culosis, certain important problems have arisen   None of these is of greater significance than the development of resistance of bacteria to this antibiotic Fortunately, an organism that has become resistant to one antibiotic does not necessarily become resistant to another   This has been well illustrated, for example, in the case of bacteria sensitive to both penicillin and streptomycin The utilization of the synergistic action of two antibiotics in order to eliminate completely all the cells of a given bacterium has, therefore, been suggested That this is possible for certain antibiotics active against gram-negative bacteria has already been demonstrated (Waksman and Reynolds, 1947)   Its application

TABLE 6

Relative bacteriostatic effect of streptomycin and streptothricin on streptomycin-sensitive and streptomycin-resistant strains of Mycobacterium tuberculosis var hominis

STRAIN	SENSITIVITY TO STREPTOMYCIN	INCUBATION	μg/ml of antibiotic required for growth inhibition*	
			Streptomycin	Streptothricin
		days		
Avirulent 607	Sensitive	7	0 2	0 8
H37Rv	Sensitive	14	2 0	8 0
H37Rv	Resistant	14	>10,000	600 0

* The inoculum consisted of 0 01 mg per ml of cell material

to the treatment of experimental tuberculosis has also been indicated (Smith and McClosky, 1945)

The proper combination of two antibiotics or of one antibiotic and one chem ical antiseptic was found to result in the killing of more of the bacterial cells than did either of the agents alone   This synergistic effect holds true only for cases where the cells of an organism made resistant to one agent still remain sensitive to the other

To determine whether this holds true for the action of streptomycin upon *M tuberculosis*, this antibiotic was first combined with streptothricin   Three cultures were used in the experiment the nonpathogenic no 607, the pathogenic no H37Rv, and the streptomycin-resistant strain of the last organism (table 6)

Both the avirulent and the virulent but streptomycin-sensitive strains of *M tuberculosis* require four times as much streptothricin as streptomycin for complete inhibition of growth   The streptomycin-resistant strain of the organism did not remain sensitive to streptothricin, although much less streptothricin than streptomycin was required to inhibit growth of this strain   The fact that 600 μg per ml of streptothricin were necessary to inhibit the growth of the strep tomycin-resistant strain would tend to remove streptothricin automatically from the field of practical utilization as a potential supplement to streptomycin

When streptomycin was combined with streptothricin, the effect upon *M tuberculosis* was additive rather than synergistic, i e , the use of 1 μg each of the two antibiotics per 1 ml of culture medium was equivalent to the action of 1 25 μg of streptomycin, namely to a simple arithmetical addition of the potency of the two antibiotics   In the case of a synergistic effect the second antibiotic would be expected to affect the few cells that remained resistant to the action of the first antibiotic   If that were the case, it would have taken comparatively little of the supplementary antibiotic to eliminate the cells of the pathogen from a culture and presumably also from the body of the host

Effect of streptomycin upon morphology and acid-fastness   The morphology and staining properties of an organism are always valuable criteria for the evaluation of the action of any drug on a particular organism   Since these characteristics are based upon the chemical constitution of the cell, any change brought about by a drug in the morphology of the organism or its reaction to stains is indicative of a change in the structure or metabolism of the cell

The tubercle bacilli have been described (Topley and Wilson, 1936) as rod-shaped organisms, 1 to 4 μ long and 0 2 to 0 8 μ broad, straight or slightly curved, with parallel or irregular sides and rounded ends, arranged singly or in small clumps, nonmotile, nonsporing, and noncapsulated   They stain with difficulty, but, when once stained, they are acid-fast

Of the variety of staining procedures tried on tubercle bacilli (Corper, 1926a), the Ziehl-Neelsen stain, using hot carbol fuchsin, sulfuric acid, and a methylene blue counterstain, has been found to be best   With this stain, the tubercle bacilli appear as red cells, while the non-acid-fast organisms are blue   Depending upon the age of the culture, differences in the densities of the stain taken up by the cells have been observed (Corper, 1926b)   Young cells, which are long, straight, or curved filaments, stain uniformly, shorter bacillary adult forms show metachromatic granules, and senescent forms are uniformly stained but are coccoid or very short bacilli

For the study of the effect of streptomycin on the morphology and acid-fastness of tubercle bacilli, a modification of the Ziehl-Neelsen stain developed by Alexander-Jackson (1944) was used   This modification differs from the original stain in that the counterstaining with methylene blue is followed by a process of decolorization and restaining with a mixture of acid green and acid yellow   In the resulting preparation acid-fast organisms appear red, partly acid-fast are mulberry to blue depending upon the degree of acid-fastness, and other organisms or debris are light green   This gives greater scope to the determination of the effect of an agent on the acid-fastness of a culture

The principal effects of streptomycin on the morphology of such cultures as *M avium* and *M tuberculosis* grown in Tween medium were loss of acid-fastness increase in granulation, and at times, especially in highly bacteriostatic concentrations, in shortening of the bacilli   A progressive loss in acid-fastness occurred with increasingly tuberculostatic quantities of the drug   This reaction was evident in the presence of concentrations of the antibiotic less than the bacteriostatic levels   Beading or granulation followed the same general trend as the loss in acid-fastness, however, this phenomenon did not seem to appear to any

significant degree except with concentrations of streptomycin which produced detectable bacteriostasis   Shortening of the bacilli or coccobacilli was observed only in those concentrations of the drug which were highly inhibitory to growth or were ultimately bactericidal   Chains of more than two or three bacilli and curving of the rods were found only occasionally   No thickening of the cells was observed   These morphological changes followed the same pattern with regard to the effect of the number of cells as did the other conditions of bacteriostatic and bactericidal action   For example, the smaller the number of cells, the smaller was the amount of streptomycin necessary to bring about malformation of the organisms   In concentrations of streptomycin which were only moderately bacteriostatic, all gradations of acid-fast staining were seen   Completely acid fast rods occurred among the clumps of almost entirely non-acid-fast ones   The question arises as to the nature of those organisms which did not undergo any transformation   It is possible that they are representative of the cells which are resistant or develop resistance to the antibiotic   In general, no completely acid-fast organisms were observed in strongly bacteriostatic or bactericidal con centrations of the drug

Morphological changes have been reported as being produced by such sub stances as toluene, chloroform, thymol, ether (Laporte, 1942), and aryl-sulfamides (Courmont, Morel, Perier, 1938) and by poor environmental conditions (Severens and Tauner, 1945, Vera and Rettger, 1940)   The organisms were reported to have an increasing tendency to grow in clumps and were altered in form   Gran ulation inside the cell was greater, the granules were liberated by the dissolution of the ectoplasm of Legroux   Loss of acid-fastness and progressive fragmenta tion of the free granules were also observed   All of these findings agree with the observation made on cultures under the influence of streptomycin

When *M tuberculosis* was grown in different media containing peptone, casein, beef extract, serum albumin, amino acids, inorganic compounds, or glycerol, normal acid-fast cells were found only in those media which contained the more complex forms of nitrogen   When the source of nitrogen was asparagine or ammonium citrate, the cells showed abnormalities, such as loss in acid-fastness and shortening and curving of the bacilli   The effects of streptomycin on the morphology of the tubercle bacilli in the various media were the same as those described for the cells in the Tween medium, namely, progressive loss in acid-fastness with increasing amounts of the drug, increase in beading, and shortening of the rods   In poor media the loss in acid-fastness was so great that the cells stained green, with blue- or mulberry-colored granules   Even in those cultures which appeared to contain no acid-fast organisms, the cells were still viable and regained their normal staining reaction on subculture to glycerol nutrient agar In short, the damage to the cells produced by streptomycin could not be neutralized by the presence of any of the nutrients used

SUMMARY

Streptomycin has not only a bacteriostatic but also a marked bactericidal action upon different strains of *Mycobacterium tuberculosis*

The size of the inoculum and the time of incubation are of great importance in determining the bacteriostatic and bactericidal activity of the antibiotic

In a growing culture of tubercle bacilli, there was a decrease rather than an increase in the proportion of streptomycin-resistant cells with an increase in age of the culture

When streptomycin and streptothricin were combined, their effect upon tubercle bacilli was additive rather than synergistic

The principal effects of streptomycin on the morphology of tubercle bacilli were loss of acid-fastness, increase in granulation, and, in highly bacteriostatic concentrations, shortening of the rods

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NOTES

REVERTING HISTOPLASMA CAPSULATUM TO THE YEAST PHASE

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The life cycle of the pathogenic fungus *Histoplasma capsulatum* can be completed outside the animal body through cultivation on blood agar incubated in a moist atmosphere at 37 C (Conant J Bact , 41, 563)   Many workers have found that some strains which had been cultured in the mycelial phase over long periods of time failed to revert to the yeast phase when cultivated under the conditions specified   When it was found in this laboratory that Francis' glucose cystine blood agar luxuriantly supported growth of the yeast phase of *Sporotrichum schencku* (Campbell J Bact , 50, 233), other systemic fungi known to exist in the yeast phase in human tissue were also observed on this medium Many strains of *Histoplasma capsulatum* which had been cultured in the mycelial phase for several years were reverted to the yeast phase without appreciable difficulty

The AMS modification of the medium originally devised by Francis has proved more satisfactory than any other medium used, and so its preparation is described

Veal infusion (double strength)	1,000 ml
Rabbit or horse blood	80 ml
Peptone	10 g
Glucose	10 g
Sodium chloride	5 g
Cystine or cystine hydrochloride	1 g
Agar	20 g

The agar, sodium chloride, and peptone are added to the veal infusion and heated until the agar is dissolved   The cystine is dissolved in the sodium hydroxide solution required to adjust the medium to pH 7 6 to 7 8 and then added to the base mixture   After sterilization at 121 C for 20 minutes, the agar base is cooled to 50 C and the blood and glucose solution added aseptically   The completed medium is maintained at 60 C for 3 hours, thoroughly mixed at intervals, and is then dispensed in tubes or plates

Histoplasma capsulatum can be easily cultured, harvested, and utilized in bacteriologic techniques when maintained in the yeast phase of growth   Several serial transfers may be necessary for the reversion of old stock strains that have been maintained in the mycelial form   Such strains should be transferred serially to new slants of the medium at 2- to 3-day intervals even though no

reversion is apparent   After 3 to 7 transfers, the appearance of small yeast colonies will be noted among the predominating mycelial types   By proper colony selection, the pure yeast phase can be secured   Once reverted, the organ ism can be maintained in this phase indefinitely by incubation at 37 C   During the reversion process, old slants should not be discarded, but should be contin uously incubated at 37 C and observed at daily intervals for papillate colonies in the yeast phase

THE VIABILITY OF YEAST CULTURES PRESERVED UNDER MINERAL OIL

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At the time that the work of Morton and Pulaski (J Bact , **35,** 163) on the preservation of bacterial and yeast cultures appeared, we were concerned with the maintenance of a large number of stock cultures of yeasts used in the prepara tion of experimental wines (Henry   Univ Wash Bull , 1937)

Briefly, the method consists of inoculating agar slants in the usual manner, incubating until good growth is obtained, and adding sufficient sterile mineral oil to cover the tip of the slant with at least one-fourth inch when the tube is upright   The cotton stopper is replaced and the culture kept at room temper ature   The culture medium used for our yeast cultures was a wort agar of the following composition   malt extract, 100 g, water to make 1,000 ml, and agar, 17 g   The pH falls to about 4 5 without adjustment

In October, 1939, 17 cultures of yeasts used in the preparation of European and American wines and a large number of unidentified strains isolated from various fermenting fruits were preserved by the method of Morton and Pulaski In October, 1940, these cultures were transplanted to a similar medium and again covered with sterile mineral oil   This second set of cultures was stored at room temperature and left undisturbed until December, 1946, when the 17 cultures of wine yeasts and a random culture from each of the 8 groups isolated from fermenting fruits were examined and subcultured for viability

All cultures appeared in good condition and many showed a white to tan, filamentous growth extending out from the slant into the mineral oil   Upon microscopic examination this growth was found to be made up of pseudomycelium and some single cells   Upon subculture on wort medium, growth appeared within 24 hours in most cases, and all subcultures showed abundant growth in 48 hours   Microscopically the cultures showed little or no mycelial growth and agreed with the descriptions made 7 years previously

REPORTED SALMONELLAS FROM THE PACIFIC
1941-1946

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During the war years several types of *Salmonella* were reported in Japanese and English journals from the Pacific area Several of these have been indexed in various abstract journals

Salmonella chiba XXVIII — —, *S nipponbasi* (XXVIII) XXXIV z_{23} —, and *S yodabasi* ? ? ? were reported by Ohasi (Japan J Exptl Med , **24,** 1431) These strains have been thoroughly studied and none of them conform with the accepted biochemical pattern established for the genus *Salmonella*   *S chiba* is nonmotile and utilizes salicin with the production of acid and gas   *S nipponbasi* produces indole and is methyl-red-negative   Its H antigen is not agglutinated by single factor z_{23} serum   *S yodobasi* conforms closely to the biochemical pattern of *Escherichia coli* and fails to grow on all selective media designed to inhibit the growth of *E coli*

S handa III,X,XXVI e,h 1,w   was reported by Ohasi (Japan J Exptl Med , **27,** 1110) and *S taihoku* III,X,XXVI l,w   1,5   was reported by Kunimoto and Tukitari (Japan Med , **3372,** 422) The antigenic pattern of *S handa* was found to be as reported and identical with the pattern of *S meleagridis* reported by Bruner and Edwards (Am J Hyg , **34,** 82) The true antigenic pattern of *S taihoku* was also found to be identical with that of *S meleagridis*

A new type, S *singapore* VI,VII k e,n,x   was isolated from cases reported as acute enteric fever by Hayakawa in 1944   This type has not been reported previously

S iwo-jima, VI,VIII 1 1,5 , *S oahu,* IV,V,XII   1,v   1,2,3 , and *S saipan,* III,X,XXVI z_6 1,6   were reported by Lindberg and Bayliss (J Infectious Diseases, **79** 91) Of these three types, the strain reported as *S iwo-jima* has been available for study in this laboratory Upon antigenic analysis, confirmed by Edwards, this strain was determined to have the pattern VIII,XX 1 z_6 , which is the pattern of *S kentucky* reported by Edwards (J Hyg , **38,** 306) *S saipan* was reported as having factor z_6 as a phase 1 antigen for the first time, and it is unfortunate that a strain is not available for further study

It is suggested that new types within the genus *Salmonella* should be verified by independent laboratories before publication in order to avoid further confusion in the classification of the *Salmonella*

ANAEROBIC FERMENTATION OF MANNITOL
BY STAPHYLOCOCCI

JAMES B EVANS

Laboratory of Bacteriology and School of Nutrition, Cornell University, Ithaca,
New York

Mannitol fermentation has long been considered an important test in studying staphylococci, and yet reports on its correlation with other tests vary widely Hallman (Proc Soc Exptl Biol Med , **36**, 789) found a 91 per cent correlation with the coagulase test on 487 strains   On the other hand, Plastridge *et al* (Storrs Agr Expt Sta , Bull 231) found that 74 per cent of their 211 coagulase negative strains fermented mannitol   Colwell (J Bact , **37**, 245) reported that of 28 mannitol-fermenting strains only 2 would ferment anaerobically   Un fortunately, her tests were not correlated with the coagulase test

Study of a small collection of mannitol-fermenting staphylococci has revealed that the anaerobic fermentation of mannitol correlated 100 per cent with the coagulase test (see table)

	NO OF CULTURES	FINAL pH IN MANNITOL BROTH	
		Anaerobic	Aerobic
Coagulase (+)	11	(10) 5 0–5 6 (1) 6 2	4 9–5 2
Coagulase (−)	21	7 0–7 2	5 0–5 9

The group of coagulase-positive cultures included 4 from clinical infections, 2 old stock cultures, and 5 from frozen foods   The coagulase-negative strains were all from frozen foods

It would seem from this that the incorporation of mannitol in selective aerobic plating media is useful, but not perfect   In addition, it has been noted that when 7 5 per cent NaCl is included in the plating medium (Chapman J Bact , **50**, 201) the acid production by coagulase-positive strains is reduced, but not, in a majority of cases, that of the coagulase-negative strains   This is so pronounced that, if bromcresol purple is substituted for the phenol red indicator, the co agulase-positive strains produce very little or no yellow zone   Of the 21 co agulase-negative strains, 14 produced a pronounced yellow zone on this medium

It is recognized that only a small collection of organisms from a limited number of sources has been used, and it is scarcely to be expected that the correlation between the coagulase test and anaerobic fermentation of mannitol will remain perfect, but the importance of anaerobiosis should be noted   This may serve to re-evaluate the importance of mannitol fermentation in studying staphylococci

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

NEW JERSEY BRANCH

New Brunswick, New Jersey, March 13, 1947

Preliminary Observations on the Growth Requirements of Bacillus popilliae Dutky and Bacillus lentimorbus Dutky S R Dutky, U S Department of Agriculture, Agricultural Research Administration, Bureau of Entomology and Plant Quarantine, Moores town, New Jersey

Bacillus popilliae Dutky, causal agent of type A milky disease of Japanese beetle larvae, has been maintained under continuous cultivation on artificial media for a year Some media gave consistently high yields of vegetative forms, but none was adequate for sporulation Cultures carried through numerous transfers produced typical disease symptoms and abundant spores when injected into Japanese beetle larvae Factors affecting culture yields were carbohydrate content, pH, buffer capacity, reducing capacity, and thiamine content of the medium Glucose and fructose served as sources of energy, whereas peptones, sucrose, lactose, galactose, or glucosamine did not About 250 million cells were produced per mg of glucose fermented B popilliae made best growth at pH 7.5 in highly buffered strongly reducing media Thiamine was essential, about 0 003 μg per 10 ml of medium supplied enough for good growth Preliminary tests indicated that B popilliae may be useful for the assay of minute amounts of thiamine

Bacillus lentimorbus Dutky, causal agent of type B milky disease, was cultured similarly

SOUTHERN CALIFORNIA BRANCH

Los Angeles, California, March 18, 1947

Anaerobic Hydrogen Transfers Affecting Fatty Acids William D Rosenfeld, Scripps Institution of Oceanography, University of California, La Jolla, California

The bacterial dehydrogenation of saturated and unsaturated monocarboxylic acids has been followed by means of the Thunberg technique Lipoclastic anaerobes isolated from petroliferous materials rapidly dehydrogenated formic acid, whereas the oxidation of other volatile acids was sporadic Saturated acids ranging from valeric through stearic were not attacked, nor were the unsaturated acids undecylenic, oleic, linoleic, and linolenic Obligately anaerobic sulfate-reducing bacteria obtained from marine muds dehydrogenated a wider range of fatty acids, although their activities were restricted to compounds containing even numbers of carbon atoms Unsaturated C₁₈ acids were also dehydrogenated

The reduction, or hydrogenation, of fatty acids was demonstrated by means of a reverse Thunberg method in which leucomethylene blue was oxidized in the presence of compounds activated as hydrogen acceptors Complete recoloration of the dye did not occur, a circumstance probably related to the comparatively high Eh level required for such oxidation Both saturated and unsaturated acids were susceptible to reduction by lipoclasts This group of acids extended from C_2 through C_{18} Reduction products were not identified

Oleic, linoleic, and linolenic acids were activated as hydrogen acceptors in the dehydrogenation of formic acid The reactions were measured in Warburg respirometers Molar concentrations of the unsaturated acids added were inversely proportional to their degrees of unsaturation, while the concentration of formic acid remained constant The amount of carbon dioxide produced in all cases approached 20 per cent of that which would result from the complete oxidation of formic acid

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PHENOL PRODUCTION BY MARINE BACTERIA *David M Updegraff*, Scripps Institution of Oceanography, La Jolla, California

Over 90 per cent of 67 samples of marine sediments examined have yielded enrichment cultures that actively decompose *l*(−)tyrosine, either as the free amino acid or combined in Difco neopeptone or casein, with the production of either phenol or *p* cresol, or occasionally mixtures of the two

The reaction proceeds well under anaerobic conditions and also when air is present over the surface of the medium, but not when air is bubbled through the medium In from 16 to 24 hours' incubation at 27 C, a medium containing 0 025 per cent of *l*(−)tyrosine and 0 1 per cent peptone in sea water at pH 7 1 to 8 2 yielded over 50 per cent of the theoretical amount of phenol, assuming that one mole of tyrosine yields one mole of phenol In a medium containing 0 025 per cent of *l*(−)tyrosine as the sole organic carbon source, an equally good yield of phenol was obtained in 48 hours Phenol is formed more often from free tyrosine when some access to air is permitted *Para* cresol is formed more often from casein or neopeptone under anaerobic conditions

Several pure cultures capable of producing phenol from tyrosine have been isolated They are all motile, nonsporulating, gramnegative rods, 1 to 1 5 by 1 5 to 5 microns They form round entire colonies with no pigment on agar Some cultures will grow on tyrosine as the sole organic carbon source None produce phenol from *p*-hydroxybenzoic acid, thus differing from *Escherichia coli-phenologenes*

THE ANTAGONISTIC EFFECT OF BACILLUS CEREUS *R J Goodlow, C W Johnson, and M V Shafer*, Department of Bacteriology, University of Southern California, Los Angeles, California

A strain of *Bacillus cereus*, isolated from milk, exhibits marked antagonistic activity against both gram positive and gramnegative bacteria Pour plates of proteose peptone agar were prepared by adding test organisms in a dilution of 300,000,000 bacteria per ml After the medium had solidified, *B cereus* was streaked across the surface of the plates Cultures were incubated at 30 C and observed at intervals of 6,

12, 24, 48, and 72 hours After a period of 6 hours' incubation, marked zones of inhibition of growth occurred in cultures of *Corynebacterium pseudodiphtheriae, Salmonella anatum, Salmonella typhi-murium, Salmonella paratyphi, Shigella ambigua, Serratia marcescens, Neisseria catarrhalis, Staphylococcus aureus, Bacillus subtilis, Bacillus mesentericus*, and *Bacillus mycoides* At the end of 24 hours of incubation all of these organisms, with the exception of *S ambigua* and *S paratyphi*, exhibited zones of growth stimulation peripheral to the zone of growth inhibition This was especially marked in *B mycoides*, which developed, after 72 hours, four alternate zones of inhibition and stimulation After 48 hours of incubation, plates of *Shigella flexneri* I and *Mycobacterium* sp showed excellent zones of growth inhibition adjacent to the *B cereus* colonies

No inhibition of growth occurred in plates seeded with *Proteus vulgaris* and *Pseudomonas aeruginosa* when cultivated in the presence of *B cereus*

Preliminary work indicates that at least one inhibitory substance is present in the filtrate of proteose peptone broth cultures of *B cereus* The antagonistic effect of *B cereus* and the isolation of the inhibitory substance seem worthy of further investigation, especially because of the inhibitory activity against the genera *Salmonella* and *Shigella*

ANTIBACTERIAL ACTION OF HEXENOLACTONE *James W Bartholomew and Francis L Hervey*, Department of Bacteriology, University of Southern California

Hexenolactone has been found to have bactericidal, bacteriostatic, and inhibitory action against such organisms as *Staphylococcus aureus, Bacillus subtilis, Serratia marcescens, Escherichia coli, Shigella paradysenteriae* var *sonnei, Shigella paradysenteriae* Flexner V, *Salmonella paratyphi, Salmonella schottmuelleri, Proteus vulgaris*, and *Pseudomonas fluorescens* Bactericidal concentrations ranged from 1 100 to 1 400, bacteriostatic concentrations ranged from 1 200 to 1 1,000, inhibitory concentrations ranged up to 1 3,200

The gram-staining properties of the organisms did not correlate with the effective

ness of the drug, and the presence of 10 per cent serum did not markedly reduce the antibacterial action For some organisms, the higher dilutions resulted in slight stimulation of growth In several instances in the presence of serum, 1 100 dilutions were much less effective than 1 200 or 1 400 dilutions

The LD₅₀ for 13- to 15 gram white mice was approximately 5 2 milligrams, for a single dose injected into the peritoneum

BACTERIOLOGY OF SCLEROMA *Robert E Hoyt and Milton Gjelhaug Levine*

An organism has been isolated from the nose and throat of cases of scleroma (rhinoscleroma), which has the following characteristics It is a gram negative rod which forms large mucoid colonies on eosin-methylene blue agar and nutrient agar Acid is formed in glucose, maltose, mannitol, and occasionally in sucrose, but never in lactose This organism is not found normally in the nose or throat and does not correspond to any other bacterium described in the bacteriological literature It is similar to organisms previously described in scleroma by a few scattered workers Etiological evidence published elsewhere by us indicates that this organism may rightly be called *Klebsiella rhinoscleromatis*

THE NATURE, PROPERTIES, AND TOXICITY OF SUBTILIN, AND ITS CHEMOTHERAPEU-TIC EFFECT ON THE COURSE OF EXPERIMENTAL INFECTIONS IN ANIMALS *A J Salle and Gregory J Jann*, Department of Bacteriology, University of California, Los Angeles, California

The antibacterial product, subtilin, obtained from the cells of a certain strain of *Bacillus subtilis*, was found to be active chiefly against gram-positive bacteria Two notable exceptions were *Neisseria gonorrhoeae* and *Neisseria catarrhalis*, both gram negative but also antagonized by the antibiotic Acid-fast organisms, including *Mycobacterium tuberculosis*, were also found to be susceptible to the antibiotic

The agent showed an extremely low toxicity to embryonic chick heart tissue fragments cultivated *in vitro* Under the conditions of the test, subtilin was found to be approximately 20 times more toxic to *Staphylococcus aureus* than to chick heart tissue, a remarkably low figure for a chemotherapeutic agent

Subtilin was shown to exert a powerful *in vivo* action on a number of bacterial infections in mice and guinea pigs Animals infected with type III pneumococcus, *Bacillus anthracis*, *Streptococcus pyogenes*, and *Staphylococcus aureus* were quickly and easily cured of the infections Recovery from the infections was so spectacular that it was almost beyond belief The antibiotic produced no observable toxic reactions in the animals

WASHINGTON BRANCH

WASHINGTON, D C , MARCH 25, 1947

DEMONSTRATION OF AGGLUTINATION AND AN AGGLUTININ-"BLOCKING" PROPERTY IN SERA OF KNOWN CASES OF BRUCELLOSIS *J J Griffitts*, U S Public Health Service, Biologics Control Laboratory, National Institute of Health, Bethesda, Maryland

Immunological techniques useful in detecting sensitization to the Rh blood factor have been adapted to the examination of sera from individuals known to have had brucellosis As in certain instances of Rh sensitization, sera of some brucellosis cases lack the ability to agglutinate *Brucella* organisms, they render the antigen insensitive to the action of known agglutinins added to such serum-antigen mixtures This agglutinin-"blocking" property of serum is present in certain sera to a much greater extent than in normal serum Sera lacking agglutinins on routine tests may agglutinate *Brucella* strains when a normal serum is used in place of saline as a diluent This appeared to be true in the test tube as well as on the warmed glass plate These findings suggest that the use of such techniques may detect immunological responses in brucellosis and perhaps other diseases although the usual tests for diagnosis using saline diluent may be negative

EASTERN PENNSYLVANIA CHAPTER

CONSTITUTIONAL FACTORS IN RESISTANCE TO INFECTION THE EFFECT OF ESTROGEN ON THE PATHOGENESIS OF TUBERCULOSIS *Max B Lurie, Samuel Abramson* (U S P H Service), *and Marvin J Allison*, The Henry Phipps Institute of the University of Pennsylvania, Philadelphia, Pennsylvania

Estrogen in large doses retarded the progress of tuberculosis at the site of inoculation in the skin and the dissemination of the disease in the internal organs as compared with the progress of the disease in rabbit litter mates of the same genetic constitution and of similar hereditary resistance to the infection   On the other hand, the periodic, intravenous injection of chorionic gonadotropin, which induced successive crops of corpora lutea, accelerated the progress of the disease in the majority of highly inbred litter mates Physiologic quantities of progesterone and estradiol exerted no consistent effect on the process, nor did ovariectomy

Estrogen suppressed tuberculin sensitivity of the skin not only in animals with

active disease but also in rabbits treated with heat killed tubercle bacilli, but the sensitivity of the internal organs was not diminished Since chorionic gonadotropin also reduced skin allergy, this effect of estrogen was not the significant factor in its retardation of the disease Estrogen reduced the inflammatory irritability of the skin to unrelated noxious agents and markedly suppressed amyloid degeneration which is incidental to chronic tuberculosis Ovariectomy, chorionic gonadotropin, and progesterone did not inhibit amyloidosis Chronic estrogen treatment induced a lymphopenia and was associated with a reduction in the weight of the adrenals There is some evidence that estrogen enhances the elimination of antibodies from their depots

It is suggested that estrogen retards the tuberculous process by reducing the dissemination of the bacilli from the portal of entry, by sparing parenchymal degeneration, and by mediating the release of antibodies through the dissolution of lympho cytes

NEW YORK CITY BRANCH

COLIFORMS WITH COMPLETE SALMONELLA ANTIGENS, OR LACTOSE-FERMENTING SALMONELLAE? *Ivan Saphra and Erich Stigmann*, Beth Israel Hospital, New York

In a previous communication the authors reported on "A coliform bacterium with the complete antigen of *Salmonella newington* " The organism isolated from a fatal meningitis in a 6-month-old baby grew on SS plates like a colon bacillus, fermented lactose, was H₂S-negative, and had the antigenic formula of *Salmonella newington* It split off a white growing variant, which also fermented lactose on subculture and produced H S Through papilla formation it gave rise again to the original coliform Both types were serologically identical This observation provoked a discussion

with two points of view evolution of a *Salmonella* out of a normal *Escherichia coli*, or haphazard variation of a *Salmonella* resulting in the splitting off of lactose fermenting substrains

An almost identical phenomenon was observed recently Again a culture with the antigenic pattern of *S newington* was isolated, this time from the stool of a man ill with gastroenteritis It grew on SS plates with the typical appearance of a *Salmonella*, on subculture, however, it fermented lactose with little gas and formed H-S abundantly In further transplants the white colonies produced red papillae These red mutants decomposed lactose rapidly with acid and much gas, they failed to produce H₂S So far this observation

parallels the older one This time, how-
ever, a third variant was found, a typical
Salmonella, lactose negative for 3 weeks
All three variants, the coliform, the inter-
mediate, the *Salmonella*, were serologically
identical Again it is impossible to ascer-
tain whether *S newington* was the end prod-
uct of a variation or the source of a retro-
gressive variation At any rate, the first
finding is no more a unique curiosity, the
repetition, again observed under natural
conditions, perhaps indicates its general
biologic importance

CHEMICAL AND IMMUNOLOGIC STUDIES OF
  LOW RAGWEED POLLEN EXTRACT *H
  S Baldwin, A W Moyer, and P F
  deGara*, Cornell University Medical Col-
  lege and New York Hospital, New York
  The immunologic activity of fractions of
ragweed pollen extract was compared with
that of standard pollen extract Fraction
B contained approximately 7 per cent nitro-
gen and 14 per cent carbohydrate, fraction
D, approximately 5 per cent nitrogen and
5S per cent carbohydrate, and fraction S,

approximately 14 per cent nitrogen and
50 per cent carbohydrate and gave a nega-
tive ninhydrin test Standard pollen ex-
tract and fractions B and D were precipi-
tated by antiragweed serum Standard
pollen extract and fraction B were also
precipitated by antifraction B serum No
precipitations were observed with anti-
fraction D serum
  Sensitization of guinea pigs to standard
extract was produced with each fraction
Sensitization to fraction B was not pro-
duced with fraction S Sensitization to
fraction D was produced with fraction D
No sensitization to fraction S could be pro-
duced The threshold of sensitivity of
untreated ragweed sensitive persons to
standard extract was lower than to the frac-
tions
  The carbohydrate fraction of ragweed
pollen extract is not an active antigen
The further the attempts to purify and
fractionate the ragweed pollen extract were
carried out, the less consistent were the
immunologic reactions observed Immu-
nologic reactivity diminished with a de-
crease in the nitrogen content

MICHIGAN BRANCH

ANN ARBOR, MICHIGAN, MARCH 27, 1947

ELECTRON MICROSCOPY AS APPLIED TO
  SOME BACTERIOLOGICAL PROBLEMS *Ruth
  Lofgren*, Department of Bacteriology,
  University of Michigan, Ann Arbor,
  Michigan
  New interest in the cytology of micro-
organisms has resulted from the develop-
ment of the electron microscope The
preparation of biological material presents
many problems Distilled water suspen-
sions of bacteria from solid culture medium
when dried on collodion-covered disks
generally make good specimens Because
of the wide variations in the sources of
organisms, techniques suitable for each
type of material must be developed
  Vital processes cannot be observed, but
consecutive preparations give similar in-
formation Studies of bacteria may clearly
demonstrate cell structures such as cell
wall and flagella Mechanical damage to
cells may give additional information, the

fibrous cell wall and granular cytoplasm
of spirochetes The effects of treatment
with immune serum, bacteriophage, chemi-
cals, etc , can be observed in detail Se-
lective staining has shown promise in some
cases The shadow casting of films reveals
surface structure or topography By com-
bining the various techniques, information
can be obtained which can contribute much
to our knowledge of microorganisms

OBSERVATIONS ON PHAGOCYTOSIS WITH
  THE AID OF DARK-FIELD ILLUMINATION
  Donald J Merchant and W J Nungester,
  Department of Bacteriology, University
  of Michigan, Ann Arbor, Michigan
  With the aid of dark-field illumination
the cytoplasmic granules of leucocytes were
seen as highly refractive bodies exhibiting
very rapid brownian motion A layer of
clear ectoplasm could be distinguished
between the granular cytoplasm and the

c

cell wall, and a clearly demarcated nucleus was observed within the cytoplasmic mass

True ameboid motion and phagocytosis were observed only in the presence of serum In its absence a random pseudopod formation occurred as well as an infrequent phagocytosis due to chance contact   The addition of serum to an inactive suspension resulted in an increased brownian motion of the granules, accompanied by a corresponding increase in the activity of the cells   A

similar stimulation was observed when the electrolyte content of the suspending me dium was lowered somewhat below physi ological concentrations

A suitable surface was necessary for the leucocytes to anchor on to form pseudopods A carefully cleaned glass surface or fibrin strands proved satisfactory   Active cells could be obtained only from animals having a high ascorbic acid level

NORTHWEST BRANCH

UNIVERSITY OF WASHINGTON, SEATTLE, WASHINGTON, APRIL 5, 1947

A REINVESTIGATION OF BACTEREMIA IN - PULMONARY TUBERCULOSIS *W F Kirchheimer*, Department of Microbiology, University of Washington School of Medicine, Seattle

Blood samples were obtained from 15 patients with far advanced pulmonary tuberculosis on three occasions at intervals of 2 weeks   Two samples were taken each time, one from the median cubital vein and one from the femoral artery   Each 5-ml sample was citrated, hemolyzed with distilled water, centrifuged vigorously, and the sediment suspended in distilled water It was incubated 1 hour at 37 C to complete the hemolysis, centrifuged, and resuspended in physiological saline to give a volume of 2 ml

This concentrate was evenly distributed into ten 2 ounce medicine bottles containing Petragnani's medium and incubated at 37 C for 4 months   All of the 900 bottles inoculated were negative for tubercle bacilli   The insensitivity of the method used or the probable intermittent character of bacteremia in tuberculosis may account for the negative findings   A test of the method made with blood containing known numbers of tubercle bacilli indicated that at least 100,000 organisms per 5 ml of blood are necessary to initiate growth on the majority of the 10 bottles inoculated

HYDROGEN PEROXIDE IN THE METABOLISM OF LACTOBACILLUS BREVIS *H C Douglas*, Department of Microbiology, University of Washington, Seattle

Suspensions of *Lactobacillus brevis* oxidize

glucose and lactate quantitatively to acetate and CO_2 without the accumulation of hydrogen peroxide   When suspensions are incubated anaerobically with lactate and hydrogen peroxide, lactate is oxidized and hydrogen peroxide is reduced according to the equation

$$CH_3 CHOH COOH + 2HOOH \rightarrow$$
$$CH_3COOH + CO_2 + 3H_2O$$
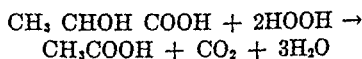
The suspensions have no action upon lactate alone anaerobically, nor upon hydrogen peroxide alone, aerobically or an aerobically   The oxidation of lactate by peroxide does not occur if the suspension is killed by heating   It is apparent that *L brevis* possesses an enzymatic mechanism for activating hydrogen peroxide as an oxidizing agent, and by definition such an enzyme would be called a peroxidase However, the reaction is not sensitive to cyanide and the usual tests for peroxidase are negative

It seems probable that in the normal oxidative metabolism of *L brevis* the hydrogen peroxide which theoretically should be formed as the first reduction product of oxygen is activated enzymatically as a hydrogen acceptor and reduced to water as rapidly as it is formed   Greisen and Gunsalus (J Bact, 45, 16) have reached similar conclusions concerning the metabolism of *Streptococcus mastitidis*

DISCOVERY OF A BACTERIOPHAGE FOR MYCOBACTERIUM SMEGMATIS *Grace M Gardner and Russell S Weiser*, Depart

ment of Microbiology, School of Medicine, University of Washington, Seattle During investigations on the isolation of bacteria antagonistic to the mycobacteria a bacteriophage for *Mycobacterium smeg matis* was encountered The enrichment method of Dubos was applied to six samples of moist leaf compost containing calcium carbonate They were incubated at 37 C for 8 months and treated semiweekly with a heavy, washed suspension of *Mycobacterium smegmatis* After 3 months tests for antagonists were begun by fixation-plating with 1 per cent glycerol agar heavily inoculated with *Mycobacterium smegmatis* Plates from two composts contained

smooth-edged plaques displaying a halo of partial lysis about a central clear zone of complete lysis

Serial Berkefeld filtrates of plaque material contained the bacteriophage in a concentration of 300 billion per ml The bacteriophage proved inactive for *Mycobacterium phlei* and a second strain of *Mycobacterium smegmatis* Its thermal death point was between 72 C and 75 C It preserved well in 50 per cent glycerol, and by lyophilization

The lack of reports of bacteriophages for the mycobacteria indicates that they may be scarce The present isolation may have succeeded because of the enrichment procedure employed

OHIO BRANCH

OXFORD, OHIO, APRIL 12, 1947

STUDIES IN HODGKIN'S SYNDROME VII CYTOPHATOLOGIC RESPONSES OF TISSUE CULTURES INOCULATED WITH AGENTS FROM HODGKIN'S DISEASE AND LYMPHOMATOSIS *Jackson W Riddle, Miriam S Flower, Margaret S Reiman, and Herman A Hoster,* Departments of Bacteriology and Medical Research, Ohio State University, Columbus

Many microorganisms have been implicated, but none have been proved, to be the etiologic agent of Hodgkin's disease Grand (1944) described the presence of giant cells and intracytoplasmic inclusion bodies in Hodgkin's tissues cultured *in vitro,* nourished with chicken embryo extract and plasma, and stained with Seller's stain Hoster, Riddle, Flower, and Reiman (1947) described a similar cytopathologic phenomenon which appears occasionally in cultures of supposedly normal chick embryo spleen, and which occurs consistently in homologous guinea pig fetal spleen cultures inoculated with cell-free extracts or ultracentrifuge preparations of Hodgkin's and lymphomatosis tissues and body fluids

Descriptions were presented of these specific cytopathologic responses Hodgkin's like cells, fuchsinophilic intracytoplasmic inclusion bodies, cytostimulation, degeneration, and alterations in the pro-

portions of the various cell types Control preparations, either inoculated, or uninoculated with preparations obtained from human sources other than Hodgkin's disease, have failed to produce these specific cytopathologic alterations

Preliminary experiments suggest that rabbit macrophage cultures may be used as substrate for the production of these inclusion bodies

A MAXIMUM DILUTION METHOD FOR THE QUANTITATIVE DETERMINATION OF PNEUMOCOCCAL POLYSACCHARIDE IN SOLUTION *Curtis Sandage and Orton K Stark,* Department of Botany and Bacteriology, Miami University, Oxford

The most accurate method for the quantitative determination of pneumococcal polysaccharide has been based on estimation of specifically precipitable antibody nitrogen This procedure is subject to all of the limitations and difficulties of interpretation encountered in precipitin tests In a study involving the preparation of pneumococcal polysaccharide it became evident that a more sensitive and more easily interpreted method might be of value

The method evolved depends on the sensitivity of mouse response to minute quantities of antigenically active polysac-

charide By proper standardization of procedures, pneumococcal polysaccharide in solution and in body fluids can be determined quantitatively in amounts not detectable by the usual precipitin tests For example, 0 00001 mg of purified SI (capsular polysaccharide) were detected by this method, although five times this amount was required to produce a positive precipitin test

This method has been used to estimate the SI content of crude preparations, with highly consistent results This indicates that it is applicable to the quantitative determination of both crude and purified SI in solution

SOME PROPERTIES OF A MUCOPOLYSACCHARIDE ISOLATED FROM A STRAIN OF CLOSTRIDIUM PERFRINGENS *Alfred A Tytell, Milan A Logan, and Alice G Tytell*, Department of Biological Chemistry, University of Cincinnati, Cincinnati

A highly viscous, alcohol-insoluble polysaccharide has been isolated from cultures of *Clostridium perfringens* (F5022, Lister Institute) The substance is soluble in water and shows characteristic carbohydrate reactions It contains no phosphorus and less than 0 1 per cent nitrogen Spectrophotometric studies of the reactions with orcinol, phloroglucinol, and diphenyl amine indicate the presence of pentoses Reaction with naphthoresorcinol and carbozole indicate the absence of uronic acids Quantitative determination of the hydrochloric acid degradation products indicate 80 per cent recovery as furfural These results predict that the substance may possibly be a pentosan Preliminary studies indicate that the substance is not antigenic

IMMUNIZATION OF MICE WITH DYSENTERY ANTIGEN ADMINISTERED BY GAVAGE OR BY VOLUNTARY DRINKING *Merlin L Cooper and Helen M Keller*, The Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati

In the course of attempts to infect white mice by gavage with living *Shigella sonnei*

we have observed the development of immunity A high degree of immunity resulted if the mice were given large numbers of bacteria as antigen Less immunity followed administration of fewer bacteria as antigen

Six doses of 49 billion living *Shigella sonnei* administered by gavage, over 4 consecutive days, stimulated complete immunity against intraperitoneal injection of 16 to 160,000 MLD of homologous organisms suspended in sterile mucin

Three to 8 daily doses of 1 4 billion living *Shigella sonnei*, or 1 1 billion killed *Shigella sonnei*, given by gavage, stimulated a significant degree of immunity in the mice when challenged 7 days later with intraperitoneal injections of 0 5 to 1,280 MDL of the homologous organisms Three daily doses stimulated sufficient active immunity to protect at least 50 per cent of the mice and 8 daily doses afforded protection to at least 80 per cent of the mice The titer of the immunity increased with increasing number of daily doses of antigen There was no significant difference in the immunizing effects of living or killed organisms Mice which drank a killed broth culture of *Shigella sonnei* over a period of 21 days were immune against 2 to 2,048 MLD of the homologous organisms when injected intraperitoneally These mice consumed an average of 7 5 billion killed *Shigella sonnei* per day

BIOCHEMICAL STUDIES OF A SOFT X RAY MUTANT OF ASPERGILLUS NIGER VAN TIEGHAM *Violet M Diller, Alfred A Tytell, and H Kersten*, Departments of Physics and Biological Chemistry, University of Cincinnati, Cincinnati

Nutrition studies have been made on a soft X-ray mutant of *Aspergillus niger* van Tiegham The mutant is stimulated 240 per cent by biotin as against 70 per cent for the normal Hypoxanthine, inositol, and p-aminobenzoic acid stimulate the mutant 140 to 200 per cent as against 10 to 50 per cent for the control Pyridoxine, pimelic acid, riboflavin, thymine, guanine, niacin, and cytosine stimulated the mutant 40 to 75 per cent but did not stimulate the normal

Analyses of the lanthanum precipitable fractions indicated that the mutant nucleic acid content was at least 25 per cent lower (on a dry weight basis) than the normal This was confirmed by spectrophotometric data

ACCELERATED PRODUCTION OF POLIOMYELITIS John 1 Toomcu, William S Talacs, and P P Pironc, Division of Contagious Diseases, City Hospital, Cleveland Succinic acid, chlorophyll, and a synthetic preparation—succinonitrile—when injected into cotton rats (0 1 ml I C , 1 ml S Q , and 2 ml I P) in concentrations of 0 03 per cent, 0 06 per cent, and 0 027 per cent, respectively, in distilled water, conditioned cotton rats so that, when 2 to 3 weeks later they were injected intracerebrally with 0 1 ml of a 10 per cent saline suspension of Flexner's cotton rat adapted strain, there was an acceleration in the production of poliomyelitis Chlorophyll alone did not cause such an acceleration

THE EFFECTS OF MALIC AND MALONIC ACIDS ON METHYLENE BLUE REDUCTION BY BACTERIA Chester I Randles and Jorgen M Birk land, Department of Bacteriology, Ohio State University, Columbus

Attempts to demonstrate malonate inhibition of methylene blue reduction by Escherichia coli with succinate as the hydrogen donator were unsuccessful Concentrations of malonate as high as 100 times that of the succinate were tried With Pseudomonas acruginosa, however, inhibition is readily demonstrable Equal concentrations of malonate and succinate (0 019 M) result in reduction times from 2 5 to 3 times longer than those with succinate alone

With certain concentrations of substrate, the reduction of methylene blue with fumarate is more rapid than with malate This is demonstrable with E coli grown on glucose synthetic media but not with P aeruginosa

P acruginosa shows much greater dehydrogenase activity for acetate, succinate, fumarate, and malate than does E coli when both are grown on glucose synthetic media However, when acetate is substituted for glucose, the activity of E coli is increased at least tenfold and is comparable to that of P aeruginosa The high activity seems to be associated with aerobic growth and indicates the intermediation of the C_4 dicarboxylic acids in acetate oxidation

EASTERN NEW YORK BRANCH

Troy, New York, April 18, 1947

A STUDY OF HEMOPHILUS PERTUSSIS BY MEANS OF THE ELECTRON MICROSCOPE Julia M Coffey and Sophia M Cohen, Division of Laboratories and Research, New York State Department of Health, Albany
A preliminary comparative study was made of Hemophilus pertussis, directed particularly to the effect of age and medium on the cell and to a possible correlation between cellular structure and antigenicity Four phase I strains were morphologically similar but different from both a pertussis strain not in phase I and two parapertussis strains Electron micrographs differentiated an outer membrane in all strains In 1-day cultures on potato-infusion rabbit-blood agar, the cytoplasm of phase I strains

was of relatively uniform density, in 2- and 3-day cultures, two types of opacity were observed—diffuse areas near the ends and clearly circumscribed spherical granules frequently centrally located Cultures in blood-free semisynthetic fluid medium had similar morphologic characters but the granules were rare and less distinct The cytoplasm of the pertussis strain not in phase I contained one or more irregularly shaped semitransparent areas, the granules were rare Hemophilus parapertussis from solid medium resembled H pertussis phase I except that the granules were commonly observed in 1-day cultures

PROPAGATION OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS IN EMBRYONATED

HENS' EGGS *Lisbeth M Kraft and Irving Gordon*, Division of Laboratories and Research, New York State Department of Health, Albany
The virus of lymphocytic choriomeningitis has been grown by others on the chorioallantois and in the yolk sac of embryonated hens' eggs This study was undertaken to determine the optimal route and incubation period for attaining high virus titer, particularly in the extraembryonic fluids, for the production of complement-fixing antigen
Eggs were inoculated via the yolk sac, chorioallantoic membrane, and allantoic sac, and various incubation periods were arbitrarily chosen Yolk sac and chorioallantoic membrane of the respective series and allantoic fluid of the three series were harvested and tested intracerebrally in mice The lethal titer of serial 10 or 100-fold dilutions of the first and fourth passage material was determined The results indicate that allantoic fluid is not a rich source of the virus but that the tissues tested contain considerable amounts (titers of 10^{-7})
Further studies are in progress using the amniotic route as well as the yolk sac and chorioallantoic routes

EFFECT OF SOIL ACTINOMYCETES AND pH ON THE MM STRAIN OF POLIOMYELITIS VIRUS *Albert Schatz*, Division of Laboratories and Research, New York State Department of Health, Albany
Culture filtrates of soil actinomycetes antagonistic to bacteriophages were tested for antibiotic activity on the MM strain of poliomyelitis virus Inoculations were made intraperitoneally in mice Under the conditions of the experiment, none of 59 preparations exerted any definite antagonistic effect on the virus Results under the conditions employed indicated the necessity for careful control of the pH of the test material

WASHINGTON BRANCH

ONE HUNDRED AND FIFTY-SIXTH MEETING, COLLEGE PARK, MARYLAND, APRIL 22, 1947

THE EFFECTS OF LACTOBACILLI ON THE QUALITY OF CHEDDAR CHEESE MADE FROM PASTEURIZED MILK *Ralph P Tittsler, George P Sanders, Homer E Walter, Donna S Geib, Oscar S Sager, and Harry R Lochry*, Bureau of Dairy Industry, Agricultural Research Administration, U S Department of Agriculture, Washington, D C
Cheddar cheese was made from milk of good quality, which was pasteurized and divided equally into two lots Lactic starter was added to one lot Lactic starter and a supplemental starter containing lactobacilli were added to the other lot The bacterial flora and chemical changes in the cheese were determined at 1 day, 2 weeks, and 1, 2, 3, 4, and 6 months
Lactobacillus casei (three types), *L arabinosus*, *L pentosus*, *L fermenti*, and *L plantarum* (including several types isolated from Cheddar cheese made from raw milk) grew rapidly in the cheese The maximum numbers of lactobacilli, from 50 to 500 millions per gram, were present at from 2 to 12 weeks, depending on the species and numbers added and on the temperature (50 or 60 F) of ripening the cheese
L bulgaricus, *L helveticus*, *L lactis*, and *L acidophilus*, added to the milk in relatively large numbers, were not detected in cheese ripened for 2 weeks or more at either 50 or 60 F The grades of the cheese were the same as those of the corresponding controls
L fermenti produced gas and a decidedly objectionable flavor in the cheese *L casei* increased the acidity in the cheese but did not increase proteolysis It increased the development of flavor but, with prolonged curing, it usually produced an acid flavor and "short" body Some strains of *L arabinosus*, *L pentosus*, and *L plantarum* increased the development of flavor and did not increase the acidity to an objectionable extent Other strains had little if any effect on the quality of the cheese

AIR SAMPLING PERFORMANCE Cretyl Crumb and B F Wells, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

In studies of airborne infection, pathogens on droplet nuclei are of greater hygienic significance than ubiquitous saprophytes on dust Therefore a standard instrument capable of efficiently sampling droplet nuclei in air is essential

The air centrifuge at $\frac{1}{2}$ cfm, at 1 cfm, slit sampler, aeroscope, sieve, and funnel recovered from 1 cubic foot of air the following number of fine droplet nuclei per fine droplet nucleus settling on a petri plate in one minute 841 5, 277 5, 385 5, 106 5, 27 S, and 1 5, respectively Against coarse nuclei the counts were 83 3, 49 5, 39 8, 23 3, 23 3, and 2 3, respectively

These results were confirmed by operating instruments in tandem The slit sampler (following aeroscope), and centrifuge (following sieve, or funnel) collected, respectively, 1 7, 47 6, and 160 1 times as many fine nuclei as were removed from this air by the preceding instruments For coarse nuclei the figures are 0 4, 0 7, and 13 3

Evidently the efficiency of sampling instruments increases with increase in particle size, and for sampling dust particles, which are much larger than these coarse nuclei, even the settling plate is satisfactory But for recovering fine droplet nuclei, one of the more efficient air samplers, such as the centrifuge or the slit sampler, is indicated

EPIDEMIC OF INFLUENZA A AMONG A RECENTLY VACCINATED POPULATION ISOLATION OF A NEW STRAIN OF INFLUENZA A VIRUS M Michael Sigel, Frank W Shaffer, and Werner Henle, The Children's Hospital, Philadelphia, Pennsylvania

In March, 1947, an outbreak of upper respiratory infection occurred in a school in New Jersey Practically all of the students had been vaccinated against epidemic influenza early in December, 1946 The epidemic was diagnosed as influenza A both by etiological and serological tests

The virus L:47 was isolated from pooled throat washings by amniotic inoculation of chick embryos It proved to be antigenically distinct from the PR8 and Weiss strains of influenza A incorporated in the vaccine Most of the individuals possessed high titers to the PR8 strain of influenza A in the acute serum specimen, indicating that the vaccine was of satisfactory potency, a fact which has been confirmed by mouse vaccination tests The acute sera showed low titers to the L:47 virus

Comparative studies on acute and convalescent sera by the inhibition of hemagglutination gave significant rises in all convalescent sera when tested with the L:47 strain The complement-fixation test with PR8 virus was as satisfactory and almost as sensitive as the inhibition of hemagglutination with the homologous virus Both tests were far superior to the inhibition of hemagglutination with PR8 or Weiss viruses which, in a number of individuals, failed to demonstrate rises in titer

A SEROLOGICAL TYPE OF PARACOLON AS A PROBABLE CAUSE OF AN EPIDEMIC OF GASTRO-ENTERITIS T F McNair Scott, Lewis L Coriell, H Davis, and Ben H Boltjes, Children's Hospital, Philadelphia, Pennsylvania

At the end of a short, mild, but extensive, outbreak of gastro enteritis at a children's camp, a bacteriological survey was made of 43 patients and 10 food handlers Two weeks after the last case, 26 of the previous patients, 33 controls, and 9 food handlers were again studied Rectal swabs revealed Paracolobactrum organisms in 28 per cent in the first survey and 15 per cent in the second Biochemically, 7 were P intermedius, 4 P aerogenoides (1 somewhat different), and 1 was different, in the first survey, in the second, 10 were different, resembling atypical Paracolobactrum or, possibly, Proteus organisms An antiserum to one formolinized P aerogenoides agglutinated the 11 similar Paracolobactrum organisms from the first survey, but none of those from

the second   Pooled 32011 antiserum behaved similarly   Antisera to 2 other first survey organisms and cross absorption tests revealed 4 antigenic subgroups with interlocking antigens one in group I, with wide antigenic crossing with the other groups, 2 in group II, 7 in group III, and 1 in group IV Representatives of the first 3 groups were agglutinated by patients' convalescent sera, but also by control sera   Further studies revealed such antibodies present in well babies and nondiarrhoeal patients in a different community, after the age of 6 months, but not before   Since antibodies from camp sera were easily absorbed by representative organisms, they possibly resulted from a wide exposure to these organisms, rather than being natural antibodies   The serological studies were conducted with O antigens   Epidemiological evidence implicated *Paracolobactrum*, 60 per cent of the organisms isolated formed a serological group and another 25 per cent were very closely related

STUDIES ON THE COMPLEMENT FIXATION ANTIGENS IN MUMPS   *Gertrude Henle, Werner Henle, and Susanna Harris,* Children's Hospital, Philadelphia, Pennsylvania

It has been demonstrated that there exist at least two serologically distinct antigens in chick embryos infected with mumps virus One is intimately linked with the infectious agent, the other is smaller in size and may be termed a "soluble" antigen Upon allantoic injection the virus-bound antigen is found mainly in the allantoic fluid, the soluble antigen largely in the allantoic membrane   The virus-bound antigen is sedimented by high-speed centrifugation at 20,000 rpm for 20 minutes, and the soluble antigen, for the greater part, at 30,000 rpm for 60 minutes   These two antigens can be differentiated by absorption of convalescent serum   Absorption with the soluble antigen leaves the antibodies to the virus bound antigen intact, and absorption with virus particles does not markedly decrease the antibodies to the soluble antigen

Upon studying sera of patients during and after an attack of mumps, and sera of people with a past history, a variable response to the two antigens has been observed   These observations suggest that for the determination of susceptibility of an individual, virus bound antigen should be employed, for the demonstration of either recent infection or contact with mumps virus the soluble antigen, and, for diagnostic purposes, both antigens will give reliable information

CONNECTICUT VALLEY BRANCH

STORRS, CONNECTICUT, APRIL 24, 1947

THE PROPHYLAXIS OF RHEUMATIC FEVER WITH SULFONAMIDES   *Nelson K Ordway,* Yale School of Medicine, New Haven, Connecticut

Twelve reports have appeared in the past eight years concerning the efficacy of sulfonamides, chiefly sulfanilamide, in preventing recurrences of rheumatic fever in individuals who have had previous attacks When severe statistical criteria are employed, it is found that only two of the papers report significant results   These two studies are, however, highly significant, and if less rigid criteria are employed, four of the other studies assume significance   Though the remaining reports fail to achieve statistical significance, they indicate without exception a reduced incidence of rheumatic fever in individuals receiving sulfonamide prophylaxis   The lowered incidence of rheumatic fever was paralleled by a reduction in the incidence of disease due to group A hemolytic streptococci and in the number of carriers of this organism   These studies are of importance, not only to the clinician in making available a tool for the control of rheumatic fever, but also to the bacteriologist in providing additional evidence in support of the now generally accepted thesis that rheumatic fever represents an allergic response to infection with the hemolytic streptococcus

A SIMPLE MEDIUM FOR GROWTH OF TUBERCLE BACILLI   *Donal L Dunphy and*

Mildred D Fousek, Yale University School of Medicine, New Haven, Connecticut
This preliminary study suggests that a medium consisting of lysed blood and glycerol supports the growth of virulent tubercle bacilli Growth is apparently more rapid than that occurring on Petragnani's medium It would seem that the amount of growth and the time requirement are in direct relation to the number of tubercle bacilli in the inoculum If this medium continues to give reliable results, its simplicity of preparation and availability are assets for its use in the diagnostic laboratory The problem of contamination can be eliminated from culture material such as sputums and gastric washings by digestion with 6 per cent sulfuric acid

THE RELATIONSHIP BETWEEN pH TOLERANCE AND VIRULENCE OF BACTERIA *J M Leise*, Department of Bacteriology, Yale University School of Medicine, New Haven, Connecticut
Virulence and pH tolerance (the ability to grow in alkaline broth) were found to be related when virulent and avirulent strains of *Shigella* and *Bacillus anthracis* were studied The virulent bacteria were able to grow in alkaline broth with smaller inocula than the related avirulent bacteria Differentiation occurred at pH 8 65 to 8 75 with the *Shigella* strains, and at pH 9 1 to 9 35 with the *B anthracis* strains The virulent strains also grew better than the avirulent in human and in horse serum These results were thought to be due to the presence of proteolytic enzymes which are more effective in alkaline solution in the virulent than in the avirulent bacteria, for the pH tolerance of an avirulent *B anthracis* strain was increased by adding trypsin to alkaline broth Also, filtrates of broth cultures of virulent bacteria showed more proteolytic enzyme activity than did filtrates of the avirulent bacteria
It was postulated that the proteolytic enzymes are associated with virulence by being related to the invasiveness (ability to grow in the body) but not to the toxicity of the organism

EXPERIMENTAL INFECTION OF FLIES WITH

HUMAN POLIOMYELITIS VIRUS *Joseph L Melnick and Lawrence R Penner*, Section of Preventive Medicine, Yale University School of Medicine, New Haven
Nonbiting flies at epidemics of poliomyelitis have been found to harbor the virus of this disease regardless of whether they have been collected at rural, suburban, or urban areas It is important to answer the question of the survival of the virus in the fly, especially as it pertains to possible multiplication in this host It would appear that one should test fly species with feeding habits that make them most likely to be contaminated with virus in nature and that one should use strains of virus which appear in nature
Human poliomyelitis virus, as naturally present in stools of poliomyelitic patients, has been fed to blowflies, *Phormia regina* After this feeding, virus was found in the flies for 2 weeks, and in their excreta for 3 weeks
Murine adapted strains (Lansing and Y-SK) of poliomyelitis virus and Theiler's TO strain of spontaneous encephalomyelitis of mice behave like biologically inert carmine in flies (*Phormia regina, Phaenicia sericata*, and *Sarcophaga bullata*) Following their ingestion by flies they may be found in gradually decreasing quantities for a period of 5 days

DETERMINATION OF BACTERIAL SENSITIVITY TO STREPTOMYCIN IN THE SMALL HOSPITAL LABORATORY *Kenneth N Atkins, and Eleanor Hoag*, Department of Bacteriology, Dartmouth Medical School, Hanover, New Hampshire
Parallel experiments with the army dilution method and paper disks containing 5, 10, 25, 50, and 100 units of streptomycin placed on surface-inoculated blood agar plates showed comparable results The disk method appears to be preferable for routine tests because of its simplicity
Of the organisms tested, *Pseudomonas aeruginosa* is unique in that by the disk method hemolysis is inhibited by the 10-unit disk though the growth is inhibited only by the 50- and 100-unit disks This observation may be a clue to the mechanism of hemolysis by this organism

KENTUCKY-TENNESSEE BRANCH

BOWLING GREEN, KENTUCKY, APRIL 26, 1947

ALCOHOLIC FERMENTATION UNDER REDUCED PRESSURE *M C Brockmann,* Joseph E Seagram and Sons, Inc , Louisville, Kentucky, *and T J B Stier,* Department of Physiology, Indiana University, Bloomington, Indiana

After inoculation with a distillery type yeast, a glucose yeast extract KH$_2$PO$_4$ medium was maintained at 30 C under an absolute pressure close to the vapor pressure of water and at the same time sparged with water vapor Control cultures, which were held at atmospheric pressure, were flushed with tank CO$_2$ at the time of inoculation in order to reduce the oxygen tension of the medium to a level comparable to that in the low pressure cultures

The pressure differences did not have a marked influence either on the rate of glucose utilization or on the ultimate yeast population Under reduced pressure the concentration of alcohol in the medium never exceeded 0 65 g per 100 ml, whereas free acetaldehyde was depressed to almost one-half the concentration found in comparable control samples In low pressure cultures 5 5 to 6 0 g of glycerol were formed per 100 g of glucose metabolized, on the same basis control cultures produced 3 0 to 3 5 g With each type of culture, glycerol formation was a linear function of glucose utilization throughout the greater part of the observation period However, under low pressure the output of glycerol per unit of yeast population per hour fell in the latter part of the observation period to approximately the same level as the control

THE OXIDATION OF CARBOHYDRATES BY A SURFACE STRAIN OF PENICILLIUM NOTATUM *Frederick T Wolf,* Department of Biology, Vanderbilt University, Nashville, Tennessee

This study is concerned with measurements of the oxygen consumption of a surface strain of *Penicillium notatum* (NRRL 1249), using the Fenn differential respirometer The QO$_2$ of *P notatum,* as measured in the lactose corn steep medium in which the fungus was grown, varies with the age

of the culture, increasing to a value above 16 mm^2 per hr per mg at 3 to 4 days, and decreasing rapidly thereafter

Glucose, galactose, mannose, maltose, and cellobiose are rapidly oxidized by *P notatum* Glycerol, calcium lactate, arabinose, xylose, rhamnose, fructose, sucrose, lactose, dextrin, mannitol, sorbitol, dulcitol, and adonitol are more slowly oxidized Trehalose and soluble starch were not oxidized, under the conditions employed, by this strain of *P notatum* The significance of the findings in relation to commercial penicillin production was discussed

A SURVEY OF THE POTABILITY OF WELL WATERS IN CENTRAL KENTUCKY *Rafael A Carlin and R H Weaver,* Department of Bacteriology, University of Kentucky, Lexington, Kentucky

Water samples have been examined from 73 wells in central Kentucky, representing an area of four counties Of these, 62 yielded coliform organisms Fifty of the wells could be classified as heavily polluted, as judged by the number of coliforms in the samples Of the 11 samples that did not yield coliforms, several gave relatively high 37 C standard plate counts This may be interpreted as indicating potential danger

Central Kentucky is a limestone region The relative lack of potable water supplies in this region confirms the common finding that strata of limestone are poor filtering agents for ground water supplies The wells that were included in the study varied from 25 to over 2,000 feet in depth and w located in various strata of limest· Except for one 2,000 foot well, no cc tion could be found between the · the wells or the strata in which located and the potability of the The soil type that overlaid the w ing strata also did not appear to nificant factor

A RAPID METHOD FOR THE Γ BACTERIAL CONTAMINATI· MERGED MOLD CULTURES

S L Adams, and W H Stark, Joseph E Seagram and Sons, Inc, Louisville, Kentucky

The rapid detection of bacterial contamination is of great importance in submerged mold amylase propagation Amylase yields are reduced if contaminating bacteria are present The use of solid or semisolid media for detecting contaminants is unsatisfactory because mold growth masks bacterial colonies and the granular nature of the inoculum makes the detection of pin point colonies difficult A turbidimetric method was developed after noting that the mold, *Aspergillus niger*, produced a pellicle but no turbidity in an enriched yeast extract glucose peptone medium The contaminating organisms, however, produce a marked turbidity in the medium in 24 hours or less

This method has been used with a high degree of success in detecting the presence of contaminating bacteria in both laboratory and pilot plant submerged mold amylase propagation In most cases, results can be obtained in 6 to 18 hours, and in no instance has it taken more than 24 hours to detect easily the presence of contaminating bacteria

TEXAS BRANCH

THE USE OF BEEF SERUM AS A DILUENT FOR CHICK MEMBRANE SMALLPOX VACCINE *Patti Crain*, Biologics Division, Texas State Health Department, Austin, Texas

The one undesirable aspect of chick membrane smallpox vaccine as produced by this laboratory since 1939 has been its lack of thermostability Following reports by Buddingh and others on the protective effect of inactivated serum, lots of vaccine diluted with inactivated beef serum, inactivated beef serum with one unit of penicillin per ml, equal parts beef serum and glycerol, glycerol saline, and a control lot of calf lymph were compared These vaccines, in capillary tubes, were stored at 37 C, room, and refrigerator temperatures, and were tested at intervals on rabbits by two series of intracutaneous titrations, by vaccinations, and by Leake and Force titrations The lots of vaccine diluted with inactivated beef serum consistently retained activity longer

After 18 months' use, a comparison between it and the old glycerol saline vaccine, on the basis of 32,000 reports from health officers throughout the state, shows that the old vaccine was giving an average of 76 45 per cent "takes," whereas the new has given 94 66 per cent "takes"

MYCOMYCIN—A NEW ANTIBIOTIC PRODUCED BY A MOLDLIKE ACTINOMYCETE ACTIVE AGAINST THE BACILLI OF HUMAN TUBERCULOSIS *Edwin A Johnson and Kenneth L Burdon*, Department of Bacteriology and Immunology, Baylor University College of Medicine, Houston, Texas

A contaminant on a Sabouraud's agar plate was found to be inhibiting strongly the surrounding growth On isolation this organism proved to be a moldlike actinomycete of unusual properties It showed a sharply limited pH growth range, failing to multiply at pH 7 0 or above Simultaneous inoculation of slanted media with various test organisms revealed a marked antagonism against several of the gram positive and gram negative pathogenic bacteria, including mycobacteria, yeast, and molds

Filtrates of pure cultures in tryptone starch broth, and also the aqueous solutions ("mycomycin") obtained after ether or amyl acetate extraction, had a similar activity The best extracts to date have completely prevented growth of the routine test organism (*Bacillus subtilis*) in dilutions of 1 7,500, and have stopped the growth of virulent human tubercle bacilli in approximately a 1 5,000 dilution The presence of serum did not greatly reduce the activity However, erythrocytes showed a definite adsorption curve over a 24 hour period Highly active concentrates are nontoxic for mice

THE DESTRUCTION OF HYALURONIC ACID BY CAPSULATED GROUP A STREPTOCOCCI *Robert M Pike and Nadine Salem*, Department of Bacteriology and Immunology, Southwestern Medical College, Dallas, Texas

When mucoid strains of group A streptococci are grown in serum broth, hyaluronic acid accumulates in the culture fluid as the capsules disappear from the cells After the maximum concentration of hyaluronic acid is reached, it remains constant during continued incubation in cultures of some strains, but in others it was found to disappear in from 1 to 7 days This decrease in hyaluronic acid concentration appears to be due to an extracellular enzyme, since sterile filtrates show the same decrease as whole cultures, but filtrates heated at 60 C for 30 minutes retain their hyaluronic acid The enzyme produced by one strain will also destroy hyaluronic acid produced by another strain These observations indicate that capsulated group A streptococci, as well as the noncapsulated strains previously described by others, may produce hyaluronidase The enzyme activity of capsulated strains, however, is relatively weak and highly variable The relation of this enzyme to the disappearance of capsules from the cells and to phase variation is not yet apparent

ALTERING DRUG RESISTANCE OF BACTERIA WITH BACTERIAL EXTRACTS *Orville Wyss*, Department of Bacteriology, University of Texas, Austin, Texas

Sterile purified nucleoprotein extracts were prepared from a drug-sensitive strain of *Escherichia coli* and a drug-resistant strain derived from it The addition of the nucleoprotein extract from the sensitive strain to a young growing culture of the resistant strain resulted in a culture in which the number of highly resistant organisms was reduced Conversely, the addition of the extract from the resistant strain to a growing culture of the sensitive strain quantitatively increased the distribution of resistant forms in the resulting population The nucleoprotein extracts were separated into the nucleic acid and protein components The nucleic acid was the active fraction

A FACTOR TOXIC TO BRUCELLA ABORTUS IN SOME LOTS OF TRYPTOSE *V T Schuhardt and L J Rode*, The Brucellosis Research Project of the Clayton Foundation and The University of Texas, Austin, Texas

Three of 7 lots of Difco tryptose tested showed the presence of a factor which specifically suppressed the growth of inocula of 5 strains of *Brucella abortus* containing up to a billion or more viable organisms The factor was not active against strains of *Brucella melitensis*, *Brucella suis*, or six other bacterial species tested The factor in 2 per cent tryptose was shown to be brucellacidal in 48 hours against inocula of 400 to 500 organisms per ml The toxic factor in tryptose broth is neutralized by blood, serum, Difco agar, and aqueous extracts of a number of plant and animal tissues This fact limits the practical significance of the toxicity factor, but we believe that the factor may possess considerable biological significance and that efforts to determine the chemical nature of the factor are justified

CYTOCHEMICAL MECHANISMS OF PENICILLIN ACTION

III. Effect on Reaction to the Gram Stain in Staphylococcus Aureus[1]

JEAN DUFRENOY AND ROBERTSON PRATT[2]

University of California College of Pharmacy, The Medical Center, San Francisco 22, California

It has been shown by application of appropriate reagents to standard agar penicillin assay plates after different periods of diffusion of penicillin that—SH groups and dienols are present outside the zones of inhibition, but are relatively scarce or are lacking inside the zones (Dufrenoy and Pratt, 1947)   The results indicated that a threshold effect involving an—SH⇌S—S equilibrium exists at the boundaries of the inhibition zones   Our conclusions, based on the macroscopic evidence obtained by developing assay plates with reagents for—SH groups and for dienols, emphasized the significance of the sulfhydryl radical as an important part of a redox system and the possible relation of sulfhydryl groups to the mechanism of action of penicillin on susceptible organisms   Our second paper (Pratt and Dufrenoy, 1947a) correlated the macroscopic patterns on the "developed" assay plates with cytochemical changes induced within the test organisms by a bacteriostatic concentration of penicillin

In the present paper we will present cytochemical evidence that correlates the bacteriostatic activity of penicillin against *Staphylococcus aureus* with changes in reaction to the gram stain and with each of the three cell constituents cited by Henry, Stacey, and co-workers (1943, 1945, 1946), as playing an essential role in a positive reaction to the gram stain, viz , (1) nucleoproteins, (2) arginine, and (3)—SH groups

Convergent lines of evidence obtained by the use of various histochemical and cytochemical techniques indicate that, previous to inhibition, cells of *S aureus* exposed to bacteriostatic concentrations of penicillin go through a climacteric period of enhanced metabolism, during which they consume sulfhydryl compounds more rapidly than they can restitute them (Dufrenoy and Pratt, 1947)   Thus a depletion of active components of aerobic respiratory systems ensues   This may be assumed to result in failure of the supply of energy required for active absorption of solutes (Pratt and Dufrenoy, 1947a)   A similar correlation is suggested by the work of Gale and Taylor (1946), who showed that bacteriostatic concentrations of penicillin block the absorption of the essential metabolite, glutamine, by *S aureus*   Their observation that the first physiologically evident effect of penicillin is inhibition of glutamine assimilation appears extremely significant, since glutamine is a component of glutathione, the activity of the —SH group of which is known to depend markedly on the vicinal NH groups

Therefore it seemed desirable to study the distribution of amino acids in the various regions of penicillin assay plates after different periods of exposure to penicillin   The work was limited somewhat in scope because, obviously, techniques that require the use of heat could not be used, since the agar would have melted, and reagents which promptly hydrolyze an agar base could not be used

MATERIALS AND METHODS

The techniques employed in this work were similar to those reported previously (*loc cit*)   The only changes were in the reagents that were used   These are described under the individual experiments   *Staphylococcus aureus* NRRL 313 was used throughout

It should be understood that the phrase "16-hour plates" refers to penicillin assay plates treated as prescribed by the Food and Drug Administration for the standard cylinder plate assay (similar to the recommendation of Schmidt and Moyer, 1944)   The phrase "3-hour plates" refers to plates that were seeded, then incubated for 3 hours before the addition of "penicylinders," and then were subjected to a second period of incubation, during which period penicillin was permitted to diffuse from the cylinders   The details of the preparation of such plates are given by Goyan, Dufrenoy, Strait, and Pratt (1947)

EXPERIMENTS AND RESULTS

Development with reagents for amino acids   (1) Sakaguchi test for arginine   It is stated that this reagent when prepared according to the directions of Glick and Fisher (1946) may be considered specific for the detection of arginine in living cells   When assay plates incubated with penicillin for 3 hours, as in the 3 hour cylinder plate assay method, are treated with the α-naphthol, hypobromite, and urea reagent, a positive reaction (manifested by development of red color) occurs in the areas of noninhibition, whereas the areas of inhibited growth develop a very faint pink color

(2) Alloxan test for α-amino acids   When 3-hour plates are flooded with a 1 per cent solution of alloxan in alcohol, a strong positive reaction (manifested by development of a deep red color) promptly appears in the background of normal uninhibited growth, whereas a very weak reaction (faint pink color) develops in the areas of inhibited growth, owing to the staining of the original colonies developed during the period of preliminary incubation without penicillin   It should be pointed out that although the alloxan-positive reaction may be considered as indicating the presence of amino acids, it has also been suggested that such a reaction might also indicate the presence of —SH groups (Serra, 1946)

(3) Millon's reagent for tyrosine   The phenolic amino acid, tyrosine, and its phenolic derivatives are held to be responsible for the very sensitive Millon reaction (Everett, 1946, p 374)   When a drop of this reagent is allowed to spread at the margin of a zone of inhibition, on a standard 16-hour assay plate seeded with *S aureus*, the actively growing microorganisms at the outer margin of the inhibition zone give a very strong positive reaction within a few minutes so that the clear inhibition zones appear surrounded by transient vivid red ·

It should be noted, however, that we do not claim specificity for tyrosine in the site of the positive Millon's reaction, especially since the region giving the strongest positive reaction with this test corresponds with that shown previously to be richest in phenolic compounds (Dufrenoy and Pratt, 1947)

Sulfhydryl groups in the gram-positive complex In 1944 Bartholomew and Umbreit pointed to "the involvement of sulfhydryl groups in the over-all gram reaction" and in 1945 Henry, Stacey, and Teece reported that gram-positive organisms differ from gram-negative organisms in that on autolysis at pH 8 and 37 C the former release some gram-positive nucleoprotein, made up of nucleic acids and of a basic protein, which in its native reduced state involves —SH groups Historically, it is interesting to note that Bach and Delétang (1931) and Delétang (1932) observed that organisms fixed in oxidizing killing fluids tend to lose their gram-positiveness, and that even earlier Deusen (1918, 1923) showed that gram-positives could be converted into gram-negatives by a number of methods He concluded that the change was the result of chemical processes These observations can now be interpreted in terms of oxidation of the sulfhydryl groups

Our experimental results show that cells of *S aureus* rapidly lose their gram-positive staining reaction when exposed to bacteriostatic concentrations of penicillin In other words, within the inhibition zones on standard 16-hour plates, where no positive reaction for sulfhydryl groups can be obtained, cells of *S aureus* are no longer gram-positive Cells picked from the uninhibited background of such plates, however, show the usual range of reaction to the gram stain that may be expected from a culture of that age, i e , actively growing typical gram-positive cells are coexistent with senescent cells which have more or less lost their gram-positive reaction Within the inhibition zones, the only gram-positive cells are those from the few penicillin-fast organisms that are constantly encountered on assay plates However, practically all of the cells which persist within the inhibition zones fully decolorize under the same treatment that preserves the stain in the majority of cells outside the zones The background of uninhibited growth on 3-hour assay plates consists of actively growing colonies, composed almost exclusively of gram-positive cells

Within the range of diffusion of bacteriostatic concentrations of penicillin, however, there can be observed all stages of loss of gram-positiveness It is notable that cells affected by penicillin, as they are in the process of division, swell into "diplococcuslike" units, and the gram-positive material appears as inclusions in two sharply defined regions, one toward each pole Each of these gram-positive regions is homologous to the portions of the cells previously described as staining vitally with neutral red under a comparable stage of inhibition by bacteriostatic concentrations of penicillin (Pratt and Dufrenoy, 1947a) Those bodies have a strong affinity for various basic dyes, such as methyl green, and may be supposed to contain, besides the phenolic compounds previously alluded to, some nucleic derivatives The next section, therefore, pertains to tests designed to reveal the distribution of nucleic acid derivatives in the areas of normal uninhibited growth and of inhibited growth on the test plates

Development with reagents for nucleic acid   The following dyes have been recommended as reagents for the detection of nucleic acid   methylene blue, Nile blue, toluidine blue, bromcresol purple, methylene green, malachite green, and safranine O   When the 3-hour assay plates are flooded with an aqueous solution (5 mg per L) of any of these dyes, a sharp definition of the inhibition zones is obtained   These reagents agree in locating the boundary of the zone at the same distance (within the limits of experimental error) around a cylinder from which a given concentration of penicillin has been permitted to diffuse (Pratt and Dufrenoy, 1947b)

DISCUSSION AND CONCLUSIONS

The data obtained by the adaptation of different histochemical and cytochemical staining techniques to penicillin assay plates are recorded in this paper and its two predecessors in the series   The present discussion will embody the results of all three papers and is intended as a résumé and summary of our work to date on the subject

A penicillin assay plate, with its zones of inhibited and of normal uninhibited growth of the test organisms, may be regarded as a field of distribution of different chemicals   The pattern of that distribution may be regarded as representative of the distribution of the several constituents and metabolic products and by products of cells that are growing normally and of cells that are under the influence of penicillin   Therefore, the addition of suitable reagents and careful observation of the reactions that occur in different parts of the test plates might be expected to impart information concerning the mechanism of the action of penicillin on the test organisms   The patterns that develop may be regarded as the result of the interaction of biological "forces," represented by the growth of the test organisms, and of physical "forces," represented by the diffusion of penicillin   The most apparent manifestation of this interaction is the development of zones of inhibition that are readily seen without further treatment on standard 16-hour plates, or that may be revealed easily by proper development with appropriate reagents on plates seeded with organisms and subjected to the diffusion of penicillin for periods as short as 3 hours   Similar chemical evidence may be obtained on standard 16-hour plates, and on 3-hour plates, although the results are sometimes obscured on the former because of the virtually complete destruction and lysis of the cells of the test organism

The first series of experimental data furnished evidence for a threshold effect involving sulfhydryl groups and, correlatively, dienol groups at the boundaries of the inhibition zones   Inhibition zones on standard 16-hour plates, and on properly developed 3-hour plates, are surrounded by a ring of maximum positive reaction for —SH groups or for dienol groups   Such an intense reaction for —SH groups may be taken as indicative of either an active synthesis of proteins or an active denaturation of proteins that results in the unmasking of bound —SH groups   In other words, an intense positive reaction for —SH groups reveals the site of intense activity of proteinases that may operate in the building up of nucleoprotein complexes or in the denaturation of such complexes

The rings surrounding the zones of inhibition are also sites of strong positive responses to Millon's reagent, probably indicating the presence of tyrosine, correlative to richness in proteins   The rings of enhanced growth are also the sites of strong positive reactions for phenolic compounds, and for nucleic compounds

Cytochemical studies of cells of *S aureus* taken from different regions of the assay plates showed that exposure to bacteriostatic concentrations of penicillin not only tends to prevent cell division, but also effects changes in the location and distribution within the cells of "vacuolar material" responsible for the absorption of vital dyes and, by extension, presumably of other solutes as well This hypothesis is in accord with the published data of other authors and our own unpublished observations on the localization of reduced silver in normal and in inhibited cells of *S aureus* following immersion in solutions of silver nitrate, exposure to light, and subsequent development   Vital staining demonstrated that the location of the vacuolar material in cells under the bacteriostatic influence of penicillin corresponds with the site of positive reactions for phenolic compounds (as shown by the reduction of osmic acid or silver nitrate and the adsorption of dyes, such as safranine or basic fuchsin) and with that which stains with basic dyes (such as malachite green and methyl green) that are known normally to stain nucleic acids

Vendreley and Lipardy (1946) describe the bacterial cell as loaded with ribonucleic acid but surmise that it is located mostly in the cytoplasm which adsorbs basic dyes strongly   The most striking change that we have observed in cells of *S aureus* affected by a bacteriostatic concentration of penicillin is sharp localization of the absorption or adsorption of basic dyes to the vacuolar material, and sharp restriction of the gram-positive staining reaction to that vacuolar material   This observation may be of fundamental significance, since, as was pointed out above, Gale and Taylor (1946) showed that cells of *S aureus* under the influence of bacteriostatic concentrations of penicillin may be considered as starving for glutamine, and since it was shown by Stearn and Stearn (1930) that "starving bacteria *gradually* lose gram-positivity passing through a stage where they present a stippled appearance with gram-positive granules throughout "   In our experiments with *S aureus*, we have observed that, under the influence of inhibiting concentrations of penicillin, the gram-positiveness of the cells fades out as the vacuolar material loses its other characteristic properties, namely, positive reaction for dienols and the ability to retain solutes

A completely satisfactory physiological interpretation of the mechanism of the action of penicillin on susceptible organisms, however, cannot be based solely on these simple observations involving evidence for the loss of the gram-positive reaction   Any attempt to correlate sensitiveness to penicillin with staining reaction must meet the objection that the gram-negative neisserias are penicillin-sensitive   It should be recalled, however, that using a modification of the gram method, Verhoeff (1940) found he was "able to stain meningococci, in spite of the fact that they are gram-negative," and that Meyrick and Harrison (1942) developed a counterstain for use in the gram technique, whereby 'the

gonococcus stains a much deeper color than any other gram-negative organism of this type usually present in smears " A final and ultimate explanation must await a comparison of chemical distribution patterns obtained with various test organisms on assay plates and a general survey of the reactions of gram negative organisms to penicillin, and especially a thorough investigation of the potentiation of penicillin action toward gram-negatives through the concomitant effect of methionine and threonine, such as has been reported by Schwartzman (1944, 1945, 1946)

In the work that has been reported in this paper and its two predecessors, color reactions obtained in various regions of assay plates and in different parts of the bacterial cells have been interpreted in terms of physiological activity The possible interference of physical phenomena such as adsorption effects and surface effects or reactions with constitutents of the agar has not been overlooked, however (Dufrenoy and Pratt, 1947) It is recognized that the over all results from experimental data of the type we have presented express the inter action of physiological phenomena with physical phenomena which can be studied separately in terms of surface effects, differential adsorption, metachro matic staining, etc Although such phenomena have not been discussed in this work, they have been given sufficient consideration and examination in our laboratory to show that they do not prevent the recognition of the physiological events reported above

SUMMARY

Cytochemical and histochemical techniques have been applied to penicillin assay plates according to methods described in previous reports

Cells of *Staphylococcus aureus* under the influence of bacteriostatic concen trations of penicillin gradually lose their positive reaction to Gram's stain

The loss of gram-positivity is correlated with changes in the character and distribution of vacuolar material and with the previously reported shift of —SH to S—S at the threshold at the boundaries of the zones of inhibition

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SOIL BACTERIA SIMILAR IN MORPHOLOGY TO MYCOBACTERIUM AND CORYNEBACTERIUM[1]

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When Lehmann and Neumann (1896) first proposed the genera *Corynebacterium* and *Mycobacterium*, the former was intended primarily for the diphtheria organism and the latter for the tubercle and leprosy organisms In recent years there has been a tendency to broaden both of these genera to include, on the one hand, almost any species showing morphological irregularity and, on the other, various gram-positive nonsporeformers even though showing little or no irregularity in morphology The original descriptions of these genera were very simple and included only the following essential characters

Mycobacterium Slender rods with some branching, acid-fast, colonies on agar, dry, wrinkled Type, *M tuberculosis*

Corynebacterium Rods with ends often swollen and club-shaped, banded with alternate streaks of stain, sometimes developing filaments and true branching (by implication non-acid-fast, although this characteristic is not definitely mentioned by the authors until a later edition of their book), growth on agar, soft and nonadherent Type, *C diphtheriae*

Various other characteristics have been listed by later authors for the genus *Corynebacterium*, the most important of which is the so-called "snapping division" of the cells As this feature is difficult to observe directly, it is usually inferred from the orientation of the cells as described by Kisskalt and Berend (1918), i e , a tendency to pile up in heaps, with palisade or V-form arrangement Stress on this characteristic by later authors has undoubtedly been responsible for some unwarranted broadening of the genus, as orientation of this sort can often be observed and does not necessarily indicate the type of cell division which is supposed to be characteristic of *Corynebacterium*

As a matter of fact, broadening of the two genera has taken place in several directions until they have come to overlap Moreover, each genus has had species assigned to it which seem to differ more from other species in the same genus than does the type species of one genus from the type of the other This broadening has taken place along the following lines

Mycobacterium (1) The inclusion of all acid-fast forms, whether or not branching occurs (2) The inclusion of many branching forms (Krassilnikov, 1934) whether or not they are acid-fast

Corynebacterium (1) The inclusion of a rapidly expanding group of "diphtheroids," i e , animal parasites which are gram-positive and show the type of orientation described by Kisskalt and Berend, a few of these are anaerobic (2) The inclusion of certain gram-positive plant pathogens, following the lead of

Jensen (1934)   (3) The inclusion (also following Jensen, 1934) of strongly aerobic soil bacteria or extremely irregular morphology, showing coccoid and branched forms as well as rods

The authors' interest in these genera comes from the fact that one of them (Conn, 1928) described under the name of *Bacterium globiforme* an organism which appears as gram-negative, short rods in 24-hour agar slant cultures but as gram-positive cocci after the cultures are 3 to 4 days old   Cultures of this organism have been sent to Krassilnikov and to Jensen, the former is sure it is a species of *Mycobacterium*, the latter that it is a *Corynebacterium*   The latter opinion has been indorsed by Lochhead, who, with one of his associates (Taylor and Lochhead, 1937, Taylor, 1938, Lochhead, 1940), has become one of the leading students of organisms of this type   Their impression in the matter has been summed up by Lochhead (1940) in the following words "The charac teristic *Bact globiforme* is now believed by us to represent a special group of the corynebacteria with distinctive cultural and physiological properties"

When *Bacterium globiforme* was first described, its author was not unaware of certain resemblances between this organism and either *Mycobacterium* or *Corynebacterium*, but it appeared different in so many ways from the type of either genus that the resemblances were regarded as probably superficial It was then named as a species of *Bacterium* because that genus was then re garded by the author as a grouping place for species whose relationships were not definitely understood   Since then, however, the conception of *Bacterium* has changed, and it is now usually defined so as to exclude an organism with morphology like that of the species in question

It must be remembered that when this species was first described the idea of life cycles involving changes in morphology had not been fully accepted, and it took some courage to describe an organism appearing as a gram-negative rod in one stage and a gram-positive coccus in another   Furthermore, the old ideas of monomorphism were then so persistent that it did not occur to the author to make a sufficiently intensive study of the organism to learn whether other morphological forms occurred in its life cycle

Work on organisms of this type was dropped in the writers' laboratory for several years   It has recently been resumed with the object of comparing cultures of the *Bacterium globiforme* type with strains from other laboratories that have been named as species of *Corynebacterium* or *Mycobacterium*, with the hope of learning how close the relationship between them may be

EXPERIMENTAL WORK

Mycobacterium Cultures

No extensive study was made of soil cultures that could be regarded as species of *Mycobacterium*   Four cultures, however, were obtained from Jensen labeled, respectively, *Mycobacterium coeliacum, M convolutum, M rubropertinctum,* and *M crystallophagum*   No similar organisms were found among the available collection of cultures isolated from local soils on ordinary media without special enrichment technique   An attempt was made to secure such forms by isolating

in media to which paraffin-coated pebbles were added, a technique which is regarded as favoring the development of acid-fasts, a few partially acid-fast organisms were found, but so late in the work that no careful study of them has yet been made

The four cultures obtained from Jensen all showed a slight tendency to branch, although not so much variation in morphology was observed as in the organisms to be discussed in the following pages   Three of them were acid-fast, although *M crystallophagum* was not   All four were gram-positive   They all grew on Mueller's tellurite agar (Difco dehydrated), with typical blackening   All four grew on agar with ammonium phosphate as a sole source of nitrogen, and none of them showed diastatic action on starch

The authors do not yet feel their work on this group has been extensive enough to warrant an opinion where in the scheme of bacterial classification these organisms belong   It should be remarked that certain students of the pathogenic acid-fasts (e g , Gordon and Hagan, 1936) regard soil acid-fasts as very closely related to the pathogens   Accordingly it seems quite likely that Jensen has been entirely justified in describing such forms as species of *Mycobacterium* It should be emphasized again, however, that Krassilnikov's "mycobacteria" (whose reaction to the acid-fast stain has never been described) do not seem to belong in the genus, but appear rather to be related to the types described below

Corynebacterium Cultures

In order to learn how closely the soil bacteria of the *Bacterium globiforme* group are related to *Corynebacterium*, it seemed desirable to obtain a collection of cultures that have been assigned to that genus   The following cultures, as representing what other workers think should go in the same genus as the diphtheria organism, were obtained 11 cultures of animal and human parasites of diphtheroid nature obtained from P R Edwards of the University of Kentucky, W A Hagan of Cornell University at Ithaca, H E Morton of the University of Pennsylvania, M Frobisher of the Johns Hopkins Medical School, three strains of *C helvolum*[2] and one of *C tumescens* (both soil organisms) from Jensen, and four plant pathogens that have been put in the genus—*C flaccumfaciens* and *C fascians* from W H Burkholder of Cornell University at Ithaca, *C poinsettiae* from M P Starr of Brooklyn College, and *C michiganense* obtained many years ago from Miss Bryan, then in the Department of Agriculture at Washington

Animal diphtheroids   The animal and human diphtheroids showed greatest similarity to the type of the genus, *Corynebacterium diphtheriae*   These organisms are comparatively constant in morphology, appearing generally as

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[2] Jensen regards this species as synonymous with Zimmermann's *Bacillus helvolus*, renamed *Corynebacterium helvolum* by Kisskalt and Berend   As there is no evidence that Jensen received any strain of Zimmermann's organism for comparison, it is preferred here to think of Jensen's *C helvolum* as an emendation of the earlier species which stands only if Zimmermann's original organism can no longer be identified   See description at the end of this article

rods, which are sometimes slightly wedge-shaped or club-shaped, although this morphological peculiarity is not ordinarily as pronounced as in the diphtheria organism itself   The palisade or zigzag arrangement of the cells is common, but truly branched cells have not been observed in the present investigation The organisms are ordinarily gram-positive, or if gram-variable, the tendency is for the young cells to be positive, the older ones negative   In physiology, the most striking feature is inability to grow on any synthetic medium investi gated, a fact which indicates their need of some organic form of nitrogen, or of accessory growth factors, or both   They do not liquefy gelatin or have any visi ble action on milk, but they are strong producers of acid from sugar

Soil organisms   The soil organisms (*Jensen's* cultures) proved distinctly different   Their morphological variations are greater, as they often show true branching of the cells, frequently with conidialike spherical bodies on the ends of the branches, and often with the production of larger coccoid bodies (called cystites by Jensen)   (See figures 2 and 3)   They are gram-variable, and un like the animal diphtheroids (figure 4) their tendency is for the older cells to be more strongly positive than the younger   The significance of this behavior to the gram reaction should be more carefully studied in relation to the present-day conceptions of the reaction as dependent on ribonucleic acid, but offhand one would say that there is a significant difference between species which tend to become gram-positive only in older cultures and those which tend to become *less* strongly so in older culture

Another striking difference between Jensen's "corynebacteria" and the animal diphtheroids is their ability to grow on synthetic media, with ammonium salts, nitrate, or urea as a sole source of nitrogen, and without the addition of growth accessory factors   They liquefy gelatin and digest milk, but show only weak production of acid from any sugar

These differences appear to the writers as being of sufficient significance to justify removal of Jensen's species from the genus *Corynebacterium*   Their striking morphology, however, is enough like that of *Mycobacterium* and possibly even of *Nocardia* (Jensen's *Proactinomyces*), that they should be kept close to these genera rather than included in the *Eubacteriales*[3]

Plant pathogens   The plant pathogens present a rather more complicated situation   It has long been recognized that the gram-positive plant pathogens do not fit properly in the genus *Phytomonas*, where several of them have been placed in the past, nor for that matter in any other available genus   Jensen (1934) apparently was the first to place one of them definitely in *Corynebacterium* (i e , *C michiganense*)   Dowson (1942) transferred two other species (previously *Phytomonas fascians* and *P flaccumfaciens*) to that genus   This step having already been taken by earlier writers, it was natural for Starr and Pirone (1942)

[3] It should be observed that some recent classifications (e g , in the forthcoming sixth edition of *Bergey's Manual*) place *Corynebacterium* in the *Eubacteriales*, *Mycobacterium* in the *Actinomycetales*   This arrangement, however, does not agree with the writers' opinion as to the actual relationships of the organisms   See the section of this article on taxonomic considerations
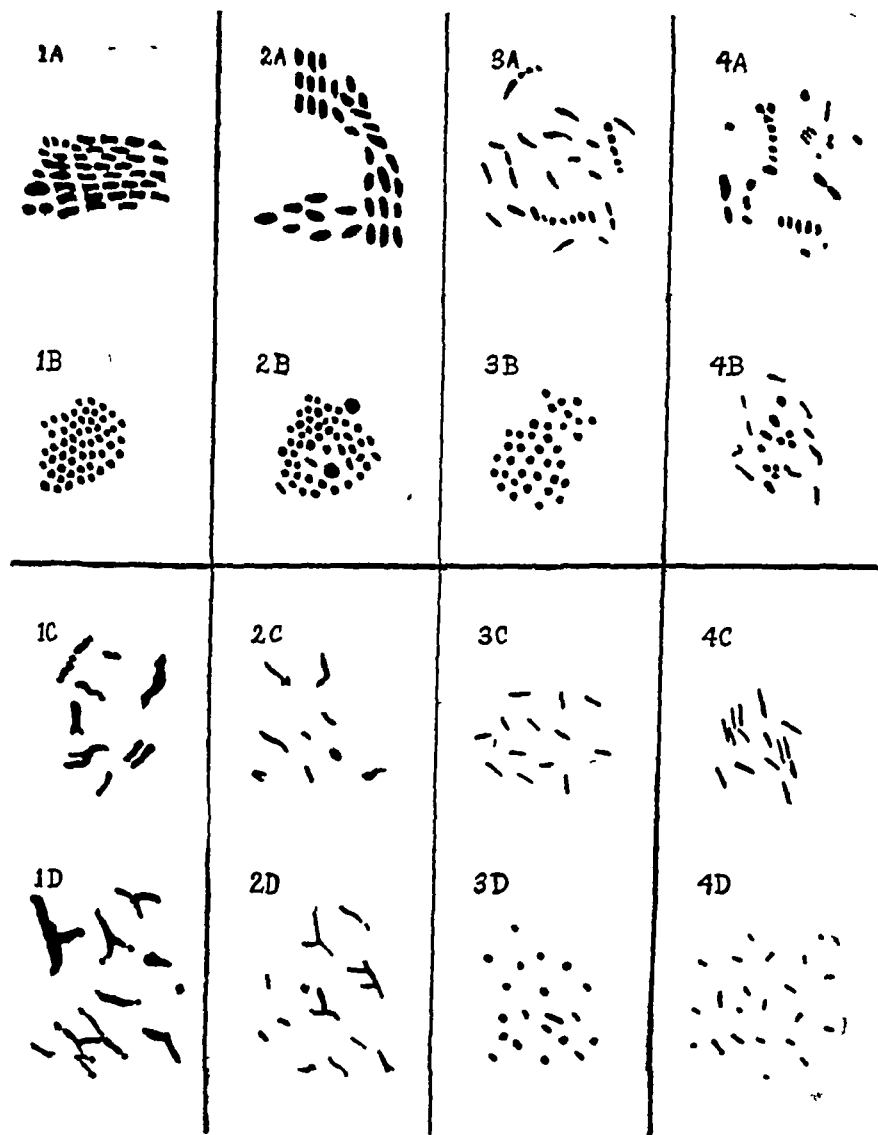
FIGS 1-4 SKETCHES TO SHOW PREVAILING MORPHOLOGICAL TYPES ON AGAR
AND IN LIQUID MEDIA, IN 1- AND 4-DAY CULTURES

Fig 1 *Arthrobacter globiforme*   Fig 3 *Arthrobacter tumescens*
Fig 2 *Arthrobacter helvolum*   Fig 4 *Corynebacterium equi*

The individual sketches are arranged in rows as follows Row A, 24-hour agar slant culture, Row B, 4 day agar slant culture, Row C, 24-hour culture in sauerkraut glycerophosphate medium, Row D, 4-day culture in sauerkraut-glycerophosphate medium Preparations shown in rows A and B stained with crystal violet, those in rows C and D with Benian's Congo red method

on describing a new gram-positive species of a plant parasite (*Phytomonas poinsettiae*) to suggest that it might also be placed in *Corynebacterium* The

present writers have obtained cultures of all four of these organisms to see whether they are related to any of the other species that have been placed in the genus, and if so to which ones

Briefly, the writers' conclusions are that all four species (with the possible exception of *Corynebacterium michiganense*) show such differences, both mor phologically and physiologically, from the type of that genus, that they clearly belong elsewhere   In fact, they show such differences among themselves that perhaps they do not all belong in the same bacterial genus   Physiologically these plant pathogens seem to stand between the animal diphtheroids and the soil forms mentioned above   they grow on synthetic media, but their proteolytic action is weak or absent   In morphology they differ so much from one another that further discussion is necessary

Corynebacterium michiganense is definitely a nonmotile, gram-positive rod, most nearly like typical diphtheroids of any of the four, although it shows but slight tendency to develop club-shaped forms or other irregularities of mor phology   Like the animal diphtheroids, it does not liquefy gelatin, and in fact shows enough similarity to the latter so that Jensen's transfer of this species to *Corynebacterium* may perhaps be justified

Corynebacterium fascians is a nonmotile, gram-variable rod, with a tendency to be more strongly gram-positive in young culture than in old   Although it shows very little morphological variation, it has an appearance on agar slant (dry and yellowish) which strongly suggests relationship to some of the *Actino-mycetaceae* (certain *Nocardia* species, for example)   As it is slightly proteolytic, it is less like typical *Corynebacteria* forms than the preceding species   Its in clusion in the genus is at least questionable

Corynebacterium poinsettiae and *C flaccumfaciens* are also yellow chromogens, they are both gram-positive, but are motile with single polar flagella   The latter species often shows sickle-shaped cells with a single, unusually long flagellum at the pole, and one would place the species unquestionably in *Vibrio* if it were not gram-positive   The inclusion of these forms in *Corynebacterium* is highly dubious   The genus is typically one of nonmotile species, and there is little, if any, justification for including types with polar flagella   Such a statement, however, need not reflect on those who proposed placing them in *Corynebac terium*, Jensen, as just shown, has good justification for transferring *Phytomonas michiganensis* to *Corynebacterium*, and Dowson as well as Starr and Pirone could point out close resemblances between that species and the other gram positive plant pathogens   Nevertheless, *C poinsettiae* and *C flaccumfaciens* are distinctly different from the typical *Corynebacterium* species, on the one hand, and from soil forms (as typified by Jensen's cultures), on the other   The present writers hesitate to say just how they should be placed, further study of the question seems indicated

Cultures from Local Soil

To compare with the above-mentioned cultures obtained from other labora tories, some 32 strains of organisms like *Bacterium globiforme* in morphology,

ısolated from local soils, were studıed   Included among them were 14 straıns
that had been carrıed ın stock for years, the rest were fresh ısolatıons   In
comparıng these with the cultures from other laboratorıes it was desired to see
whether they belonged ın *Mycobacterıum* (after Krassılnıkov) or ın *Coryne-
bacterıum* (after Lochhead and Jensen), or ıf ın neither, where they should be
placed taxonomıcally

A brıef study was enough to convınce the wrıters that acceptance of Krassılnı-
kov's conceptıon ıs out of the questıon   These cultures can scarcely be called
Mycobacterıum, chıefly because they show no evıdence of acıd-fastness   Also,
they show rather more tendency to branch than typıcal members of that genus,
ın thıs they somewhat resemble *Nocardıa* ın morphology, but differ from ıt ın
havıng smooth, soft growth on agar (lıke ordınary bacterıa) rather than the dry,
wrınkled growth suggestıve of the tubercle organısm   The fact that Krassılnıkov
called a culture of *Bacterıum globıforme* a specıes of *Mycobacterıum*, whereas
Jensen states that the same culture belongs ın *Corynebacterıum*, ıs strong evı-
dence that the former's *Mycobacterıum* ıs equivalent to the latter's *Coryne-
bacterıum*   Jensen's conceptıon seems more acceptable than Krassılnıkov's
As a matter of fact, the sımılarıty of the cultures ısolated from local soils to
Jensen's *Corynebacterıum helvolum* ıs so great that careful study was needed to
show that there really are dıstınct differences

Morphology   Practıcally all the cultures selected for thıs comparatıve study
showed the morphologıcal growth cycle on agar whıch has been descrıbed ın the
past as characterıstıc of *Bacterıum globıforme*, and ıs ıllustrated by the photo-
mıcrographs of Conn (1928, p 6) as well as by sketches 1A and 1B ın thıs paper
Brıefly, ıt may be saıd that the organısms appear as gram-negatıve rods ın 1-day
culture, and as prevaılıngly gram-posıtıve coccı ın older cultures   Thıs partıcu-
lar type of morphology ıs chıefly characterıstıc of agar slant cultures   In lıquıd
medıa the rods tend to elongate and branch, as shown ın figures 1C, 1D, and 5
These branchıng forms are most easıly shown by the Benıans' negatıve staın,
usıng Congo red turned blue by treatment wıth acıd, wıth thıs technıque the
apparent dıameter of the cells ıs smaller than when they are posıtıvely staıned
ın dry condıtıon, a difference that shows ın the sketches of figure 1   Sometımes
ın lıquıd cultures about 24 hours old, the nodes of these branched forms appear
swollen, and when a gram staın ıs made of such cells, the swellıng proves to be
due to a gram-posıtıve coccoıd body at the node, the rest of the cell beıng gram-
negatıve (see figure 5)   Accordıng to Krassılnıkov (personal correspondence)
these structures are actually germınatıng spores   In older lıquıd cultures sımılar
coccoıd bodıes seem to be borne lıke conıdıa on the ends of the branches, and ıt
can be shown that these are also gram-posıtıve   It ıs stıll uncertaın whether
both of these types of spherıcal bodıes are ıdentıcal, or whether they are the same
as the coccoıd forms whıch show ın older cultures on solıd medıa   Krassılnıkov's
ınterpretatıon of the matter, on examınatıon of cultures from thıs laboratory,
ıs that the organısm goes through a regular lıfe cycle   coccoıd arthrospores,
germınatıng forms wıth several branches radıatıng from the remaıns of the spore,
long rods wıth a tendency to branch, shorter rods, and finally by a process of

further shortening, the breaking up into coccoid arthrospores   No actual demonstration of such a life cycle has been made here, and clearly no such cycle does occur on agar where only rods and cocci are observed   Moreover, if the forms shown in figure 5 are merely germinating spores, it is difficult to explain how the remains of the spore can retain its gram-positive nature while the rods developing from it are gram-negative   Furthermore, although these forms are observed regularly in the cultures regarded here as typical of what has been called *Bac terium globiforme*, other types apparently closely related show no stages except the rods (more or less elongated and more or less irregular in shape) and the cocci   Another guess, which is probably as justified as that of Krassilnikov's, is that the conidialike bodies formed at the ends of the branches are the same as those seen on old agar slants, whereas those formed at the nodes (which are somewhat larger) are another type of spore similar to what Jensen calls cystites,
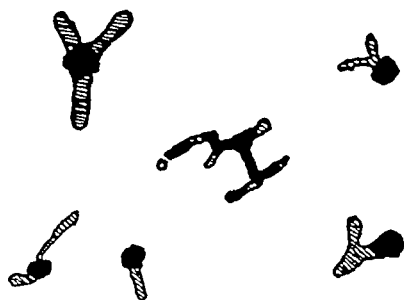
FIG 5 ARTHROBACTER GLOBIFORME, 24-HOUR CULTURE IN SAUERKRAUT
GLYCEROPHOSPHATE MEDIUM, STAINED BY THE GRAM METHOD
Gram-negative structures are shaded, gram-positive structures are solid

It will be seen from figure 2 that the morphology of Jensen's *Corynebacterium helvolum* is similar   The chief difference is the occurrence of the larger spherical bodies ("cystites") and the persistence of some rod-shaped cells in old agar cultures   Jensen's *C tumescens* is quite different (figure 3)   No other culture of Jensen's have been available to the authors, but the illustrations in his paper make it evident that *C helvolum* is the one showing the greatest morphological similarity to what has been recognized here as "*Bacterium globiforme* "   Cultures exactly agreeing with Jensen's have not been isolated from local soils

Physiology   When organisms having this type of morphology were first recognized, it was realized that they showed little difference among themselves in physiology, but as nearly all the physiological characteristics were negative ones (except gelatin liquefaction, which was always positive), it was not felt that this apparent similarity was significant   One positive characteristic was the production of small amounts of acid on synthetic media   Jensen lays considerable stress on this, in fact he regards his organisms as distinctly different from *Bacterium globiforme*, because he finds low pH values in carbohydrate media inoculated with *Corynebacterium* strains, whereas *Bacterium globiforme* has been described as producing little acid   It should further be mentioned that it was thought at one time in this laboratory that cultures of this organism could be separated into two species, one producing acid from lactose, the other failing to do so   Subsequent work has shown that none of these differences are of

significance   All of the forms under consideration, regardless of whether they are called *Corynebacterium*, *Bacterium globiforme*, or some other name, can show low pH values after growth on nonbuffered carbohydrate media   It is felt, however, that because of the small amount of actual acid (probably largely CO_2) indicated by the pH changes in the absence of buffer, and because of variations observed in the same cultures on repetition of the tests, such acid production is of no significance and is certainly of no value in the separation of species in the group

Taylor (1938) divides his cultures (all of which he regards as representatives of *Bacterium globiforme*) into two types   type I utilizes either NO_3 or urea as a sole source of nitrogen, but type II does not grow on a medium containing either of these nitrogen sources, glucose, and mineral salts   The present writers have observed no such distinction   All of the cultures they have found showing the typical morphology described above grow in media having no nitrogen other than one or the other of the two compounds in question   This either means that no representatives of Taylor's type II have been found locally or that the distinction observed by him has failed to appear under the writers' conditions   It should be remarked that among all the cultures studied here, Jensen's *Corynebacterium tumescens* is nearest like Taylor's type II, but it proves, when in vigorous condition, to be able to utilize either NO_3 or urea nitrogen

Another characteristic of the organisms that was at first thought to be of value for classification is the reduction of nitrate to nitrite   Recent investigation, however, indicates that all the organisms of this group do reduce nitrate and that nitrite production can be detected if a synthetic medium of the right consistency is employed (Dimmick, to be published)

At one time in the course of the investigation it was hoped to make use of bacteriophage typing as a means of separating species from one another in this group   This method had, in fact, proved to have value in classifying certain other soil bacteria (Conn, Bottcher, and Randall, 1945)   It did not, however, prove adaptable to the group under investigation, either because of lack of specificity in the bacteriophage, or because of easily developed resistance by the bacteria, or both   It was accordingly given up as a criterion for classification

Recent study has shown one biochemical test which may be constant enough to separate the cultures into two groups—diastatic action on starch   If this characteristic proves constant on further study, a new species must be made for those forms which do not show such action   Also there are some cultures that are yellow chromogens and that may be a distinct species   Because of the extreme variability in physiology shown by these organisms, however, no such species are made at the present time

TAXONOMIC CONSIDERATIONS

As explained above, it is felt that Jensen was mistaken in placing such forms in the genus *Corynebacterium*, because there are striking differences between these organisms and the type species of this genus (the diphtheria organism)   Morphologically, however, they show greater similarity to *Mycobacterium* and *Corynebacterium* than to eubacteria   Undoubtedly, therefore, they belong in the

Mycobacteriaceae, in spite of certain morphological resemblances to *Nocardia* (*Proactinomyces*)   There does not seem to be any genus which exactly fits them in any present system of bacterial classification

The writers propose for this purpose to revive, by emendation, an old name, *Arthrobacter* Fischer (1895), which as originally proposed was a *nomen nudum*, as no species were named and it was subsequently abandoned even by its author It is not inappropriate for the present purpose, as it was defined by Fischer as including all nonflagellate, rod-shaped bacteria which produce "arthrospores" as recognized by DeBary   Just what DeBary's arthrospores may have been is not certain, and Fischer later expressed some doubt as to their actual nature, but as the term has been recently revived as a possible name for the conidialike bodies observed in the bacteria now under consideration, an emendation of Fischer's name to apply to them seems permissible

To discuss the relation of this emended genus to *Mycobacterium* and *Corynebacterium*, certain general points of bacterial classification must be considered   In this the writers prefer to follow the classification given in the fifth edition of *Bergey's Manual*, rather than that which is to be used in the forthcoming sixth edition   This choice is made, first, because the latter classification has, at the time of writing, been distributed only in mimeograph form and, secondly, because in the grouping to be employed in the sixth edition, *Corynebacterium* is placed in the *Eubacteriales* and *Mycobacterium* in the *Actinomycetales*, and the writers prefer to regard these two genera as closely related   According to the fifth edition of *Bergey's Manual*, the differences between these groups may be defined as follows

A Simple and undifferentiated forms, without true branching   Occur as spheres, short or long straight rods, or as curved rods   *Eubacteriales*

B Cells rod-shaped, clubbed or filamentous, with decided tendency to true branching   Conidia may be formed   *Actinomycetales*

  I Rods, or filaments with only slight branching   True conidia not formed
                                                            Mycobacteriaceae

  II Filamentous forms, often branched, sometimes forming mycelia   Conidia often present   *Actinomycetaceae*

The family *Mycobacteriaceae*, as described above, may in the writers' opinion be divided into at least the following three genera

    I Aerobic slender rods, nonmotile, wholly or partially acid-fast, gram-positive, sometimes clavate or cuneate, or occasionally with rudimentary branching   Many species pathogenic to animals
                                       Mycobacterium L and N

    II Aerobic to microaerophilic rods, ordinarily nonmotile, non-acid-fast, gram-positive (most strongly so in young culture), cells often irregularly shaped, clavate, cuneate, or with rudimentary branching, often beaded or barred   Ordinarily require organic nitrogen, growth accessory factors, or both, typically animal parasites, but some dairy forms, possibly some plant pathogens[4]   *Corynebacterium* L and N

[4] If all but the animal parasites ("diphtheroids") are removed from this species, the authors can see no objection to its transfer to the *Eubacteriales*, as proposed for the sixth edition of *Bergey's Manual*

III 'Strongly aerobic forms, showing rather complicated morphological life cycles, including rods, cocci, clubs, and branched forms, non-acid-fast, gram-variable (young cells usually negative, the older cells, especially those in coccoid form, usually positive), able to live on inorganic nitrogen without added growth accessory substances, typically soil organisms

Arthrobacter, Fischer, emend

The last-named genus can be characterized as follows

Arthrobacter Fischer, *emend*

Morphology Varied, with a tendency to go through a more or less definite life cycle, the most characteristic features of which are gram-negative rods in young cultures and gram-positive coccoid forms (arthrospores?) in old cultures, with intermediate stages that may be clubs, branched forms, or short unbranched filaments Large (1 to 2 μ) spherical bodies are sometimes observed which have been termed "cystites"

Cultural characteristics Growth on surface of solid media soft and smooth, not dry and wrinkled or hard and leathery, as ordinarily in *Mycobacterium* and the *Actinomycetaceae* Colonies on poured plates ordinarily small (punctiform) Growth in broth usually slow and never profuse

Physiology Can ordinarily use either ammonium salts or nitrates as sole sources of nitrogen Can utilize glucose and sometimes other sugars as sources of carbon and energy, but ordinarily without producing sufficient quantities of acid to have appreciable effect on the pH of highly buffered media (e g , containing peptone) Gelatin usually slowly liquefied Ordinarily cause blackening of Mueller's tellurite agar

Habitat Primarily soil

Type species A globiforme (Conn) Conn and Dimmick

It seems possible at present to recognize three species

Species 1 *Arthrobacter globiforme* (Conn) *comb nov* (*Bacterium globiforme*, Conn, 1928, *Achromobacter globiforme*, Bergey *et al* ,*Manual,* 3d ed , 1930) See figure 1, A to D

Rods in young standard agar culture of fairly regular morphology, 0 6 to 0 8 by 1 0 to 1 5 μ, becoming (after 2 to 4 days) cocci of about 0 6 to 0 8 μ, branching forms with similar cocci and also large spherical bodies (1 to 2 μ) in liquid media Growth vigorous, cream colored (never lemon yellow), on standard agar or on synthetic agar with ammonium salts, nitrate, or urea as the sole source of nitrogen Diastatic action on starch agar (Further characterization as given in *Bergey's Manual*) One of the most abundant organisms in local soil

(It is possible two other species can be recognized, one differing from the foregoing species in producing lemon yellow on agar, the other in failing to show diastatic action on starch No names are being assigned to them, however, until the constancy of the differences has been proved)

Species 2 *Arthrobacter helvolum* (Zimmerman), *emend* Jensen, *comb nov* (*Bacillus helvolus,* Zimmerman, 1890, *Corynebacterium helvolum,* Zissekalt and Berend, 1918, *emend* Jensen, 1934) See figure 2, A to D

The three cultures on which this interpretation of the species is based were

secured from Jensen   It is not at all certain that they are the same as Zimmer-
man's organism   *Corynebacterium helvolum* Kisskalt and Berend, however, was
based on a culture received from Zimmerman, and it seems difficult at present to
learn just what species it may have been   The present writers, therefore, prefer
to regard Jensen's description as an emendation   Based on Jensen's cultures,
the species has the following distinctive characteristics

Morphology Similar to *A globiforme* in young agar slant culture, older cul-
tures appear as mixtures of rods, small cocci, and the larger spherical bodies,
never appearing as though a pure culture of a micrococcus, as is typically the case
with the foregoing species, in liquid media, appearance is similar to that of *A
globiforme*   Growth on standard agar, usually lemon yellow, although sometimes
merely cream color   Moderately strong to weak diastatic action on starch
This species has not been found in local soil

Species 3   *Arthrobacter tumescens* (Jensen, 1934) *comb nov* (*Corynebacterium
tumescens*, Jensen, 1934) See figure 3, A to D

Morphology on standard agar slant similar to that of *A globiforme*, but rods
in young cultures are more irregular, in liquid media the branching forms are
rare or absent   Nonchromogenic   No growth on tellurite agar   No diastatic
action on starch   Growth rather scanty on either standard or synthetic media

This species seems to be something like the type II of *"Bacterium globiforme"*
recognized by Taylor (1938), although it apparently utilizes urea and NO₃
nitrogen   It has not been found in local soil   The description is based on a
single culture obtained from Jensen

Possible Other Species

Jensen places two other species (*Corynebacterium cremoides* and *C insidiosum*)
in the same group with the last two species named, a group which is characterized
by great morphological irregularity   He claims the two species to be synonyms
of *Bacterium cremoides* Lehmann and Neumann, and of *Aplanobacter insidiosum*
McCulloch   The present writers have never received cultures of these forms and
do not know whether they should be placed in *Arthrobacter,* according to Jensen's
descriptions they seem to be closer to this genus as here defined than they do to
true *Corynebacterium*

CONCLUSIONS

There has been a tendency within the last ten or fifteen years to place certain
soil bacteria and plant parasites in the genera *Mycobacterium* and *Corynebacte-
rium,* this practice seems to have started independently with Krassilnikov and
Jensen in 1934

The present study has made it evident that Krassilnikov's *Mycobacterium* is
the same as Jensen's *Corynebacterium* and is not acid-fast   Partially acid-fast
organisms, apparently related to *Mycobacterium,* do occur in soil, but as they do
not seem to make up part of the predominant soil flora, they have not been in
cluded in the present study

Special attention has been given to forms found in local soils that are similar to

Jensen's group I of *Corynebacterium* (which show much morphological variation and which he claims are most closely related to the diphtheria organism) It is clear that among these forms should be included *Bacterium globiforme* Conn It is also evident that they differ so much from *Corynebacterium diphtheriae* that, although probably related to it, they scarcely belong in the same genus For this group of species the name *Arthrobacter*, emended from A Fischer, is here proposed, with *Arthrobacter globiforme* (Conn) *comb nov* as the type

A less intensive study has been made of the plant pathogens that have been placed in *Corynebacterium* It is concluded that *Corynebacterium michiganense* may well belong in that genus, but the inclusion there of *C fascians* is questionable, *C flaccumfaciens* and *C poinsettiae*, however, should not have been placed in it, chiefly because they are motile, with a single flagellum at one pole

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THE EFFECT OF IMPURITIES ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLIN

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The possible significance of impurities in the chemotherapeutic action of penicillin was first suggested by Cornman (1944) and by Lewis (1944), who observed that impure but not pure penicillin possessed a selective lethal action against rat and mouse sarcoma cells

Dunham and Rake in 1945 demonstrated that impure penicillin exerted a definite effect on the motility of *Treponema pallidum,* whereas crystalline penicillin had no such activity

In 1946 Smith showed that germination and root growth is retarded by impure, and not by crystalline, penicillin and stated that the indole-3-acetic acid and phenylacetic acid present in impure penicillins are responsible for this effect

In a recent communication (Hobby et al, 1946), the comparative efficacy of four forms of purified penicillin and random samples of impure penicillin was described   On a unitage basis, (CD_{90}), the relative chemotherapeutic efficacy of penicillins X, dihydro-F, G, F, and K was on the order of 500, 143, 100, slightly less than 100, and 60, respectively   The values for penicillin F were obtained with a preparation which contained impurities, the action of which was not known

All of the samples of commercial penicillin tested were three to five times more effective than crystalline penicillin G in protecting mice against experimentally produced hemolytic streptococcal infections   The protective action exerted by these impure penicillins was of the same order regardless of the unitage per milligram, or the value of the *Bacillus subtilis, Staphylococcus aureus* differential ratio

In a series of experiments carried out simultaneously with those reported in the present paper, Welch, Randall, and Price (1947) have confirmed the fact that impure preparations of penicillin are more effective than crystalline penicillin G, and in addition have demonstrated that the action of crystalline penicillin G may be enhanced by the addition of penicillin impurities

EXPERIMENTAL PROCEDURES AND RESULTS

The present study was undertaken in an attempt to confirm and extend the observations previously reported from this laboratory (Hobby et al, 1946) and to determine, if possible, the factors responsible for the greater activity of impure preparations of penicillin

Method   Throughout this study all comparisons were made by means of mouse protection tests, using the standard procedure previously described (Hobby et al, 1946)   Unless otherwise specified, *Streptococcus hemolyticus,* strain

C203Mv, or *Diplococcus pneumoniae*, strain I/230, was used as the infecting organism   One ml of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions of a 14-hour rabbits' blood broth culture was injected intraperitoneally into each of a series of 18- to 22-gram white mice   A minimum of 10 mice per dilution of culture was used in each series   Treatment was started exactly $2\frac{1}{2}$ hours after the infecting dose The penicillin was administered subcutaneously in 90 per cent peanut oil   Forty per cent of the total dose was administered $2\frac{1}{2}$ hours, 40 per cent 7 to 8 hours, and 20 per cent 24 hours after infection   Treated animals were observed for a period of 14 days   A control series of untreated animals was included in each day's experiment   The untreated infection was uniformly fatal within 48 hours in the case of *S hemolyticus*, 96 hours in the case of *D pneumoniae*

Materials used [1]   Throughout this study crystalline penicillin G having a potency of 1,634 units per mg, by the bioassay method, and a *B subtilis, S aureus* differential ratio of 1 0 was used   The polariscopic assay of this preparation was 1,635 units per mg   Ultraviolet absorption indicated 100 per cent penicillin G The crystalline penicillin K used showed a potency of 2,182 units per mg and a differential ratio of 0 36, the crystalline penicillin X, a potency of 1,069 units per mg and a differential ratio of 1 39, the purified penicillin dihydro-F, a potency of 1,675 units per mg and a differential ratio of 0 57

The impure penicillins used consisted of fractions recovered at various stages in the extraction of penicillin G   The potencies of these varied from 2 8 to 1,028 units per mg, the differential ratio varied from 0 62 to 0 92

Action of crystalline and purified penicillin in pneumococcal infections   The chemotherapeutic effect of highly purified or crystalline penicillins and impure penicillin on hemolytic streptococcal infections in white mice has been reported previously (Hobby *et al*, 1946)   In order to be certain that the difference observed between the actions of impure and purified penicillins on this organism is not characteristic of a single organism only, similar experiments were carried out on a small scale, using *D pneumoniae*, type I (strain I/230), as the test organism The results are indicated in table 1

Considering G as 100, the relative order of efficacy on a unitage basis for penicillins X, dihydro-F, F, G, and K was on the order of 302, 180, 116, 100, and 63, respectively, on a CD_{50} basis, 204, 138, 100, 100, and 26   The impure preparation tested was more effective against the experimentally produced pneumococcal infection than crystalline penicillin G   The relative efficacy of impure penicillin to crystalline penicillin G, on a CD_{80} unitage basis, was on the order of 461 to 100, on a CD_{50} unitage basis, 290 to 100   The relative efficacy of the various penicillins was closely comparable with that previously reported by ourselves

(1946) and by Eagle and Musselman (1946) for *S hemolyticus* and by Eagle (1947) for *D pneumoniae* [2]

Comparative action of crystalline penicillin G and impure penicillins in hemolytic streptococcal infections   In view of the fact that impure preparations of penicillin appeared to be more active than crystalline penicillin G, it seemed of interest to determine, if possible, the source and nature of the agent responsible for this phenomenon

TABLE 1

Chemotherapeutic action of various forms of penicillin on pneumococcal infections (type I) * in mice

FORM OF PENICILLIN	λ	DIII F	F	G	K	IMPURE	CONTROLS
Oxford units per mg	1,069	1,675	960	1,634	2 182	1,307	
$\frac{B\ subtilis}{S\ aureus}$	1.39	0.57	0 66	1 00	0.37	0 87	
Total dosage	Therapeutic effect Percentage of survival						
units							
60	62	30				80	3
100	62	70				82	
150	85		65	62		100	
210	98	80	70	72	45		
300			78	76	42		
400			98	82			
500					60		
600					82		
Relative activities							
CD$_{50}$ Biological	302	180	116	100	63	461	
Gravimetric	194	180	107	100	88		
CD$_{10}$ Biological	204	138	100	100	26	290	
Gravimetric	131	138	92	100	35		

* Strain I/230, pneumococcus type I   A minimum of 10 mice was used for each dilution in each set   One ml of a 10^{-7} dilution contained 1 to 10 lethal doses of pneumococci, 10^{-6}, 10 to 100, 10^{-5}, 100 to 1,000, 10^{-4}, 1,000 to 10,000

Crystalline penicillin G and impure penicillins from seven stages in the extraction and recovery of crystalline penicillin G were tested for their relative efficacy in the control of experimentally produced hemolytic streptococcal infections in mice   The total dosages used were 30, 60, 100, 150, 210, and 300 units

Four of the impure fractions of penicillin tested (preparations 4, 5, 6, and 7)

[2] In a previous communication from this laboratory (Hobby et al , 1946), the relative efficacy against *Streptococcus hemolyticus*, on a gravimetric (CD$_{50}$) basis, was 127, 100, 57, and 40 for penicillins X, G, F, and K, respectively   Eagle and Musselman (1946) reported 260, 100, 50, and 9 for these penicillins, respectively   Recently Eagle (1947) reported values of 160, 100, 83, and 19 against pneumococcus, whereas the data above, on a CD$_{50}$ gravimetric basis, indicates a relative efficacy of 131, 100, 92, and 35   The differences undoubtedly indicate variation in technique of injection

penicillin G to 1 mg (54 units) of this same preparation of impure penicillin was not sufficient to produce this degree of protection   The protection resulting from the combined action of 1 mg of impure penicillin containing 54 units per mg and 50 units of crystalline G was no greater than that resulting from treatment with 1 mg of this preparation of impure penicillin alone   Administration of 0 1 mg (5 4 units) of impure penicillin in combination with 50 units of crystalline G produced, however, more protection than 50 units of crystalline G alone   Whereas 50 units of crystalline G alone had given only 64 per cent protection, this amount of G in combination with 0 1 mg of impure penicillin (5 4 units) gave 78 per cent protection
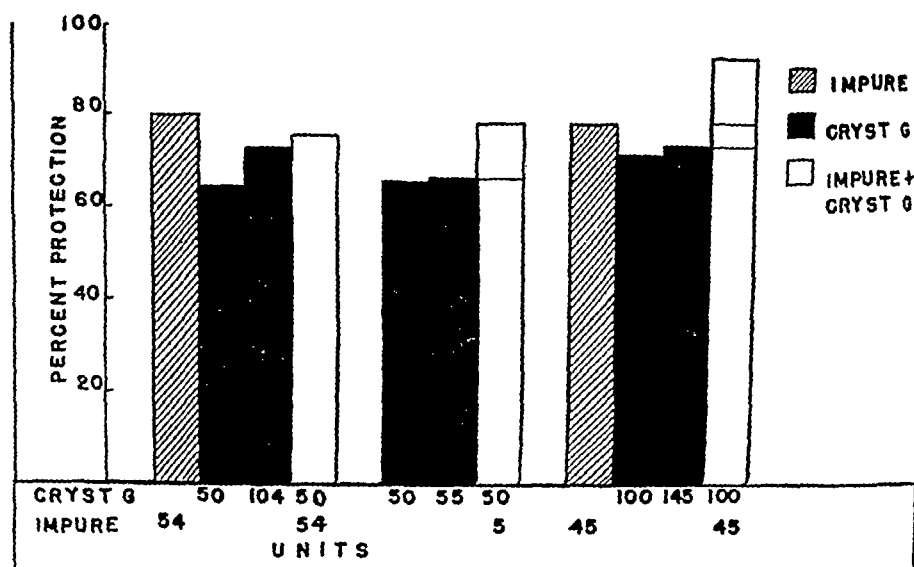
GRAPH 2 THE COMBINED ACTION OF CRYSTALLINE PENICILLIN G AND IMPURE PENICILLIN ON HEMOLYTIC STREPTOCOCCAL INFECTIONS

When 100 units of crystalline G were used, in combination with 0 83 mg of im pure penicillin, the resultant protection was again greater than could be obtained from either the amount of crystalline G or the amount of impure penicillin present   Likewise, the percentage of protection was greater than could be ob tained with amounts of crystalline G equivalent to the total amount of active penicillin present in the mixture (graph 2)

It was apparent from these experiments, therefore, that the action of crystal line penicillin G could at times be enhanced by the addition of impure penicillin

Effect of mixtures of crystalline penicillin G and inactivated impure penicillin In subsequent experiments the effect of inactivated preparations of impure penicillin on the activity of crystalline penicillin G was determined   It is recognized that penicillin may be inactivated specifically by penicillinase, which is present in certain preparations of clarase   A concentrated preparation of

penicillinase, made by acetone and alcohol precipitation of clarase,[3] was used in the majority of the experiments   One milligram of this enzyme was sufficient to inhibit completely the action of 2,000 units of penicillin   In order that an excess of penicillinase might always be present, 1 mg was used routinely for inactivation of each 1,000 units of penicillin   For inactivation, penicillinase was added to the solution of penicillin and the mixture incubated at 37 C for 4 hours   At the end of this time samples were withdrawn and tested for potency by the Oxford cup plate method   Only preparations showing, at this stage, no active penicillin were used   The mixtures were then heated at 80 to 85 C for 1 hour to destroy the penicillinase present   Mixtures were stored on dry ice until potency results were available   Samples were again withdrawn for testing, after it had been ascertained that all potency had been destroyed, and 100 units of crystalline penicillin G were added to that amount of inactivated penicillinase mixture, which would contain 0 1 mg of penicillinase   The mixture was incubated for 4 hours at 37 C, diluted, and again tested for potency by the Oxford cup plate method   Only those preparations of inactive penicillin which did not destroy any of the crystalline penicillin G were used

Varying amounts of impure penicillin inactivated by penicillinase in this manner were mixed *in vitro* with crystalline penicillin G   The chemotherapeutic action of these mixtures of crystalline G and inactivated impure penicillin was tested in mice previously infected with hemolytic streptococci, strain C203Mv   Four of the impure preparations previously described (preparations 1, 5, 6, 7, see table 2) were tested   The dosage used consisted of 100 units of crystalline G combined with amounts of inactivated impure penicillin equivalent to that weight which had contained 100 units prior to inactivation   One preparation (no 6) was also tested in amounts ranging from 0 01 to 1 85 mg (0 54 to 100 units prior to inactivation)   Likewise, 25, 50, and 75 units of crystalline G were tested in combination with 1 85 mg of this inactivated impure penicillin (preparation 6)   In view of the fact that preliminary experiments had suggested that preparation 1 was no more effective than crystalline penicillin G (table 2), larger amounts of this material, equivalent to 200 and 300 units prior to inactivation, were used

One hundred units of crystalline penicillin G, in combination with that weight of preparations 5, 6, and 7, equivalent to 100 units prior to inactivation, were sufficient to produce 90 to 100 per cent protection against hemolytic streptococci   Preparation 1, which previously had been shown to be no more effective in the active form than crystalline penicillin G, likewise was capable of enhancing the

[3] A suitable preparation of clarase, obtained from the Takamine Laboratories, was dissolved in water, 7 volumes of acetone were added, and the mixture was allowed to stand in the refrigerator overnight   The brown supernatant fluid was decanted, the precipitate was dissolved in distilled water, and two volumes of 95 per cent alcohol were added   The mixture was again cooled in the refrigerator until precipitation was complete   The mixture was then centrifuged, the supernatant fluid discarded, and the precipitate redissolved in distilled water   Alcoholic precipitation was repeated three times or until a white, flocculent precipitate appeared   The precipitate was dissolved in a small volume of water, frozen, and dried *in vacuo*   By this procedure a readily soluble and highly active preparation of penicillinase was obtained

protective action of 100 units of crystalline G to 95 to 100 per cent when weights of the inactive material equivalent to 200 or 300 units, prior to inactivation, were used (graph 3)

One hundred units of crystalline penicillin G, in combination with as little as 0 01 mg of preparation 6 inactivated by penicillinase, was sufficient to produce over 90 per cent protection against hemolytic streptococci As indicated previously, 100 units of crystalline G alone produced only 71 per cent protection (graph 4)
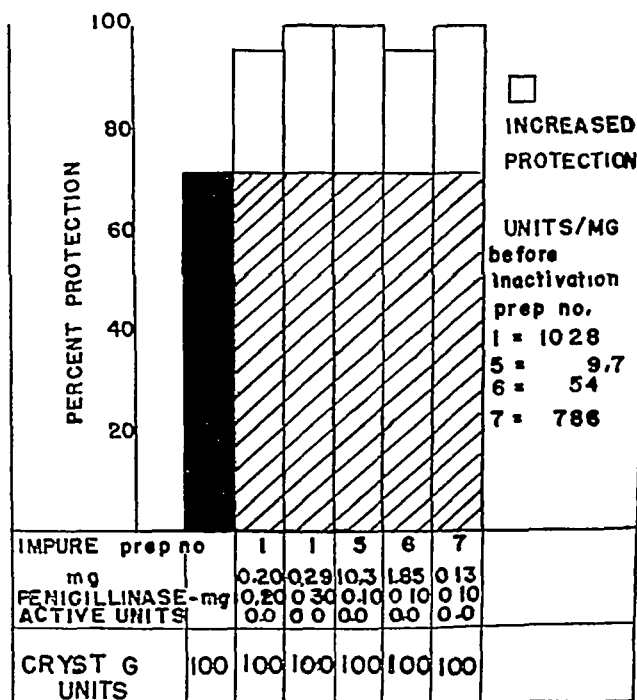
GRAPH 3 EFFECT OF INACTIVATED IMPURE PENICILLIN ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLIN G (100 UNITS)

Seventy-five units of crystalline G in combination with 1 85 mg of inactivated impure penicillin were adequate to produce 86 per cent protection, whereas 75 units of crystalline penicillin G alone would be expected to produce only 69 per cent protection Fifty units of crystalline G in combination with this amount of inactivated impure penicillin produced 72 per cent protection as compared to 62 per cent with crystalline G alone On the other hand, 25 units of crystalline G combined with this amount of inactivated impure penicillin produced the same degree of protection as would be expected with crystalline G alone The degree of protection due to the crystalline penicillin G, therefore, was increased 10 to 27 per cent by the addition of suitable amounts of inactive impure penicillin Control experiments using 1 24 to 2 68 mg of inactivated impure penicillin alone indicated that this material had no protective action itself (graph 4)

It was apparent that the protective action of crystalline G was enhanced by the addition of inactivated impure penicillin as well as by the addition of active impure penicillin

Effect of penicillin degradation products on crystalline penicillin G   In the inactivation of penicillin by penicillinase, a marked increase in the degradation products present in these impure preparations naturally occurred   Attempts were therefore made to test the effect of these substances on the activity of crystalline penicillin G *in vivo*

Crystalline penicillin G was inactivated by penicillinase, heated to destroy the enzyme, and tested in the manner previously described for impure penicillin

IMPURE PENICILLIN											
mq	0	0 01	0.10	1 00	1 85	0	1 85	0	1 85	0	1 85
units		0 54	5.4	54	100		100		100		100
PENICILLINASE(mcg)	0.54	5.4	54	100	100		100		100		100
UNITS F	0	0	0	0		0		0		0	
INACTIVATION											
CRYST G- units	100	100	100	100	100	75	75	50	50	25	25

□ INCREASED PROTECTION
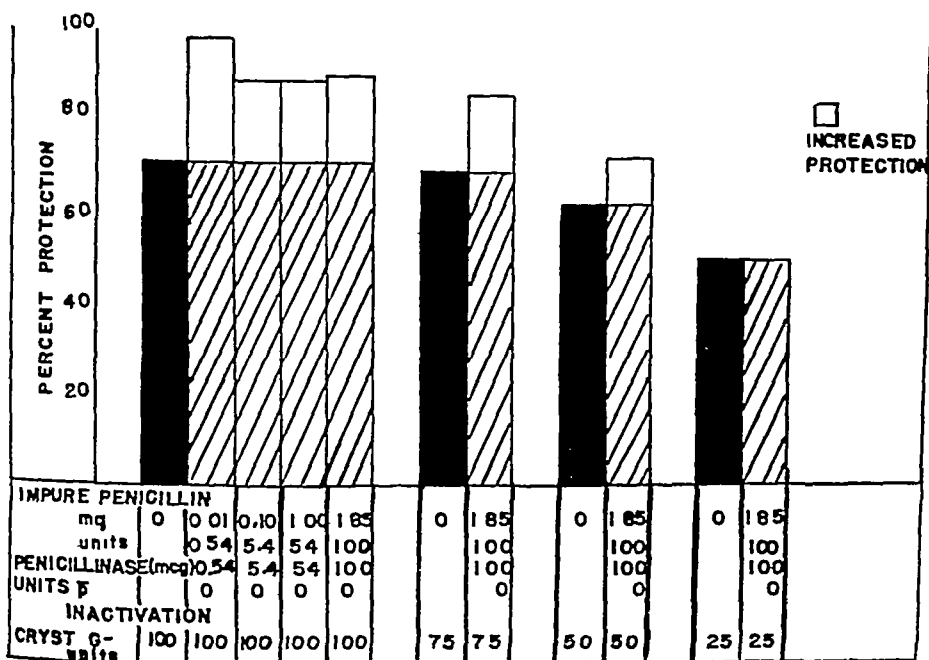
GRAPH 4 EFFECT OF INACTIVE IMPURE PENICILLIN ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLIN G

There was no indication either that any active penicillin or that any active penicillinase remained   This inactive form of penicillin was then made up to its original potency by the addition of active crystalline penicillin G and the resultant mixture tested for its chemotherapeutic action against hemolytic streptococci in mice   There was no enhancement of the action of crystalline penicillin G   Indeed, the results suggest that these degradation products may even decrease the action of crystalline G

In view of the possibility that small amounts of residual active enzyme might have been present in these experiments, further studies were carried out with crystalline penicillin G that had been inactivated by alkali   Again there was no enhancement of the action of crystalline G

Preliminary experiments with a preparation of crystalline G high in penicil linic acid have suggested that this substance not only plays no part in the en hancement of crystalline penicillin G, but may possibly decrease the efficacy of penicillin   Previous data have indicated that phenylacetic acid likewise is probably not correlated with the factor responsible for the enhancement of crystalline penicillin G *in vitro* (table 2)

No attempt has been made to evaluate the effect of each of the degradation products of crystalline penicillin G   Those formed by alkaline or enzymatic inac tivation of this form of penicillin, as well as penicillinic acid and phenylacetic acid, however, appeared to have no ability to enhance the action of crystalline peni cillin G

Effect of inactivated penicillinase on crystalline penicillin G   Since large amounts of enzyme were used in the inactivation of the impure penicillin, the pos sible effect of this substance on the activity of crystalline penicillin G was of interest   Protection studies were therefore made of mice infected with hemolytic streptococci and subsequently treated with crystalline penicillin G combined with varying amounts of the preparation of penicillinase used above   In each case the enzyme was heated at 80 to 85 C for 1 hour and then tested, before being used, to make certain that no activity remained   Dosages consisting of 100 units of crystalline G combined with 0 00054 to 0 10 mg of inactivated penicillinase pre pared from clarase and 100 units of crystalline G combined with 0 54 mg of a preparation of inactivated penicillinase made from *Bacillus* sp [4] were tested

As little as 0 054 to 0 10 mg of the inactivated penicillinase prepared from clarase was sufficient to increase the protective action of 100 units of crystalline penicillin G from 71 per cent to 88 to 90 per cent   On the other hand, 0 54 to 1 0 mg of the inactivated enzyme prepared from *Bacillus* sp enhanced the action of crystalline G to only 78 to 80 per cent   Penicillinase itself, therefore, was prob ably not entirely, if at all, responsible for the effect   The remarkable effect of small amounts of the preparation of penicillinase obtained from clarase suggested that some other substance must exist in clarase which is responsible for its activity (graph 5)

Effect of clarase on crystalline penicillin G   Clarase, lot no 2404,[5] which con tained no penicillinase, was chosen for subsequent study   Again with hemolytic streptococci as the infecting organism, mouse protection studies were made   In fected animals were treated subcutaneously with 100 units of crystalline penicillin G combined with 1 0 mg of clarase   Under these conditions 100 units of crystal line G was adequate to produce 97 per cent protection   One milligram of clarase gave no protection

It is apparent therefore that whereas the impure preparations of penicillin are more effective than crystalline penicillin G, and whereas the impurities from

[4] We are indebted to Dr A J Liebmann of Schenley Corporation for the culture of *Bacillus* sp (no 569) used   Penicillinase was prepared by acetone and alcohol precipita tion of broth cultures of this organism   One milligram of the preparation used was sufficient to inactivate 100 units of penicillin

[5] Clarase, lot no 2404, containing no penicillinase was obtained through the courtesy of Mr W A McIntyre and Dr Mildred Adams, Takamine Laboratories, Clifton, New Jersey

penicillin can enhance the activity of crystalline G, the activity of penicillin can also be enhanced by other substances not normally present in impure penicillin Concentrates of penicillinase prepared from clarase will produce such an effect That this effect is probably not due entirely, if at all, to the penicillinase itself is implied by the fact that a preparation of clarase containing no penicillinase can similarly enhance the action of crystalline G, whereas a preparation of penicillinase from another source has little effect

Effect of inactivated impure penicillin on the chemotherapeutic action of penicillins X, dihydro-F, and K In view of the fact that the action of crystalline penicillin
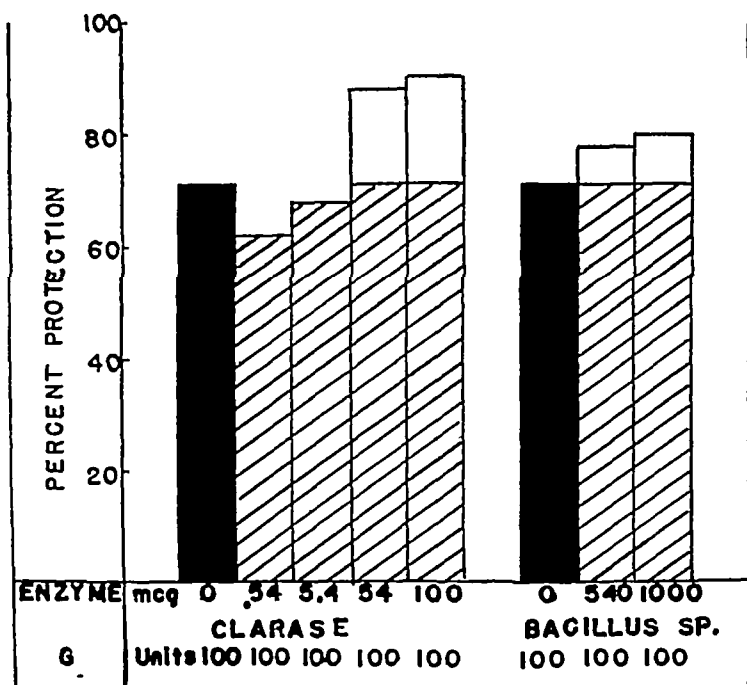

GRAPH 5 EFFECT OF INACTIVATED PENICILLINASE ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLIN G

G could be enhanced by inactivated crude penicillin preparations, and in view of the fact that impure preparations of penicillin are also more effective than the other forms of penicillin, it seemed of interest to determine whether or not one could demonstrate a similar enhancement of the activity of other penicillins by impurities

Impure penicillin, preparation 6, inactivated in the manner previously described, was again used   An amount (1 85 mg) which had contained 100 units of penicillin prior to inactivation was mixed with 100 units of highly purified penicillin dihydro-F, with 10, 23, and 40 units of crystalline penicillin X, and with 100 units of crystalline penicillin K   In addition, 3 70 mg of this inactivated preparation were also mixed with 100 units of crystalline penicillin K   These mixtures

were tested for their therapeutic action against hemolytic streptococcal infections in the usual manner

Under these conditions, 100 units of penicillin dihydro-F was adequate to protect 86 per cent of mice against infection   This preparation of dihydro-F, alone, in a dosage of 100 units, afforded only 75 per cent protection   Ten, twenty-three, and forty units of crystalline penicillin X in the presence of inactive impure penicillin gave 60, 100, and 90 per cent protection, respectively   In the absence of impurities, one would obtain only 10, 34, and 62 per cent protection, respectively, from this preparation of crystalline penicillin X   With 100 units of crystalline penicillin K, in the presence of an amount of inactivated impure penicillin which had contained 100 units prior to inactivation, only 50 per cent protection resulted   In the absence of impurities, however, this preparation of
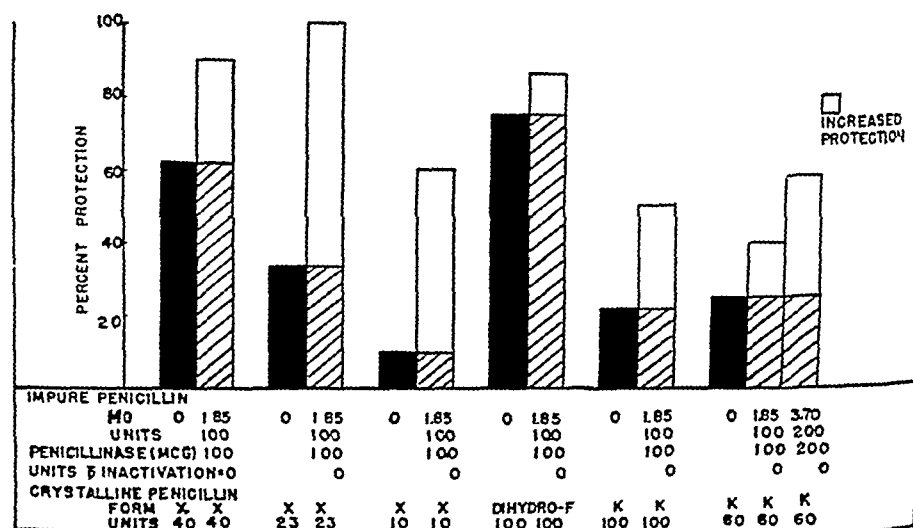
GRAPH 6 EFFECT OF INACTIVE IMPURE PENICILLIN PREPARATIONS ON THE CHEMOTHERAPEUTIC ACTION OF CRYSTALLINE PENICILLINS X, DIHYDRO-F, AND K

penicillin K, in a dosage of 100 units, gave only about 22 per cent protection Approximately 230 units would have been necessary to produce 50 per cent protection   The addition of a larger amount of inactivated impure penicillin to this preparation of crystalline penicillin K did not further enhance its effectiveness

In a subsequent series, 1 85 mg of inactive impure penicillin (preparation 6) combined with 60 units of crystalline K produced 40 per cent protection, whereas 3 70 mg combined with this amount of crystalline K gave 57 5 per cent protection   Sixty units of K alone in the strain of mice used for this particular experiment were capable of protecting only 25 per cent of the animals infected[6] (graph 6)

[6] This set of experiments was conducted with a different strain of mice from that used for previous experiments   The degree of protection due to 60 units of crystalline penicillin K in this strain was similar to that due to 100 units in the strain of mice previously used

It is apparent therefore that under suitable conditions, the action of all of the penicillins (X, dihydro-F, G, and K) may be enhanced by the presence of impurities The effect on penicillin K, however, is less regular

Action of mixtures of purified or crystalline penicillins   In view of the fact that many preparations of impure penicillin contain, in addition to impurities, a mixture of the various penicillins, further studies were carried out to determine the effect of these penicillins on each other

In preliminary experiments, a preparation of mixed penicillins recovered from impure material was used   This preparation (120) having a potency of 1,197 units per mg and a *B subtilis, S aureus* differential ratio of 0 95 was separated into two fractions (1) a fraction containing a mixture of penicillins and having a potency of 1,574 units per mg and a *B subtilis, S aureus* differential ratio of 0 97 and (2) a fraction containing predominantly impurities and having a potency of only 190 units per mg and a differential ratio of 1 03   All three fractions were tested for their chemotherapeutic activity   In a dosage of 100 units, the original preparation and the fraction containing only 190 units per mg each protected 85 per cent of the animals infected, whereas the purified preparation of mixed penicillins afforded only 46 per cent protection

Although high in penicillin G, this purified preparation contained small amounts of penicillins F, dihydro-F, and possibly K   Analyses indicated 91 per cent penicillin G—the remaining 9 per cent probably consisting predominantly of dihydro-F, with not more than 2 to 3 per cent K   The amount of penicillin X present was negligible   The amounts of these penicillins present were incapable of enhancing the action of the penicillin G

In subsequent preliminary experiments, mixtures of known amounts of highly purified or crystalline penicillins were tested   Sixty units of crystalline G were mixed (1) with 20 units of dihydro-F, 19 units of K, and 1 unit of X, and (2) with 20 units of dihydro-F and 20 units of K   The chemotherapeutic action of these mixtures was greater than would have been expected from 100 units of dihydro-F, G, or K   Indeed, it was as high as would be expected from 60 to 100 units of impure penicillin or of crystalline penicillin X   No greater protection was obtained with the mixture containing 1 per cent penicillin X, however, than with that completely devoid of X (graph 7)

The preliminary experiments described indicated that whereas a mixture of penicillins, containing dihydro-F, F, and G with a small amount of K and probably no X, had no greater chemotherapeutic action against hemolytic streptococcal infections than crystalline G, a mixture containing, in addition to crystalline G, 20 per cent dihydro-F and 20 per cent K was far more potent than crystalline G alone   Whether the latter effect was due to the penicillin K alone, to the larger amount of dihydro-F in the latter mixtures, or to the combined action of dihydro-F and K could not be determined from these data

The combined action of crystalline penicillins G and K were therefore tested in a limited series of animals   Twenty units of crystalline penicillin K were mixed (1) with 40 units of crystalline G and (2) with 60 units of crystalline G   Sixty units of crystalline penicillin G were used as control

Sixty units of crystalline G afforded 72 per cent protection,[7] whereas the com bination of crystalline penicillin G and K afforded 88 to 92 per cent protection It was apparent that crystalline penicillin K can enhance the activity of crystal line penicillin G and that the effect is greater than would be anticipated from the total number of units present

The effect of host susceptibility on penicillin dosage   In the course of the experi ments described above, an opportunity to observe the effect of alteration in host susceptibility on the penicillin dosage required to protect against hemolytic streptococcal infections in mice presented itself
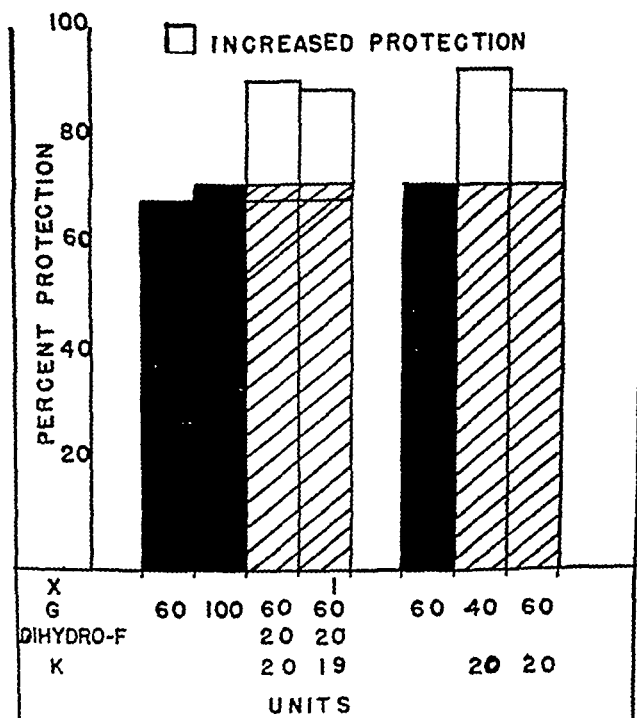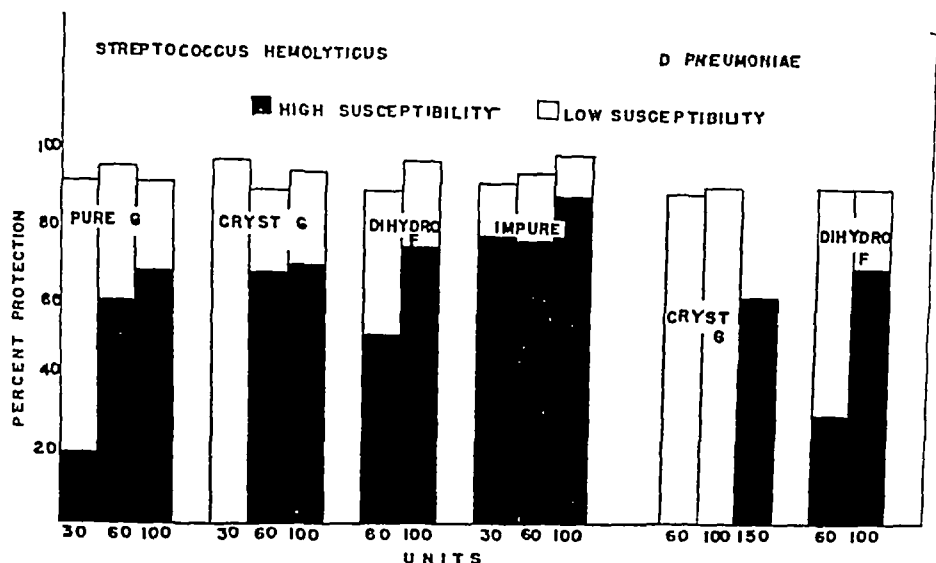

GRAPH 7 CHEMOTHERAPEUTIC ACTION OF MIXTURES OF PENICILLIN

A spontaneous infection due to an organism believed to be similar to *Corynebac terium murium*, described by Condrea (1930), developed throughout the mouse colony   This infection was benign in nature and at no time fatal to the spontane ously infected mice   Shortly after the infection was first observed, however, it became apparent that this strain of mice was no longer as susceptible to hemo lytic streptococcal and pneumococcal infections as in the past   Although the infections were still fatal to this strain of animals, death occurred slowly and, in the higher dilutions, less uniformly   Coincident with this shift in host suscepti

- Owing to the fact that a strain of mice differing from that employed in the majority of previous experiments was used here, the percentage of protection due to 60 units of crystalline G differed slightly from that previously described

bility, the amount of penicillin necessary to protect against such streptococcal and pneumococcal infections decreased markedly   Whereas 250 to 300 units of penicillin G had previously been necessary to give approximately 90 per cent protection against hemolytic streptococcal infection, 15 to 30 units of this same material were now adequate to produce this degree of protection   Likewise 60 units of penicillin dihydro-F were now as effective as 210 units had previously been, and 30 units of a preparation of impure penicillin were as effective as 100 to 150 units in the past   Similarly the amount necessary to protect against pneumococcal infections in this strain of mice was lowered (graph 8)

GRAPH 8 COMPARISON OF THE CHEMOTHERAPEUTIC ACTION OF PENICILLINS
IN MICE OF HIGH AND LOW SUSCEPTIBILITY

It was not possible during this time to demonstrate agglutinins or precipitins against hemolytic streptococci in the sera of these mice   The serum did, however, possess a marked antibacterial action against this group of organisms Zones of inhibition varying from 11 to 35 mm in size were observed with samples of undiluted sera when tested against *Staphylococcus aureus* (H) by the Oxford cup plate method used for penicillin assays   Normal sera showed no zones of inhibition   The heating of samples of whole blood to 80 to 85 C for 2 hours, during which time coagulation of the red blood cells occurred, was sufficient to prevent diffusion of the active principle, although spot plate tests indicated that it was still present in an active form   The substance responsible for the antibacterial action was stable at 56 to 60 C for 2 hours, as indicated by the fact that the size of the zones of inhibition of *Staphylococcus aureus* was not decreased

Streptococcus hemolyticus, strain C203Mv, was inhibited in broth by one sample of pooled sera from these mice in dilutions up to 1 512, whereas pneumococci were inhibited by dilutions up to 1 1,024 and staphylococci by dilutions up to 1 128

As the infection in the colony regressed, newly bred animals of the same strain gradually regained their original susceptibility to pneumococcal and streptococcal infections   Simultaneously, the amount of penicillin necessary to induce protec tion approached the original levels

In view of the fact that a shift in susceptibility sufficient to alter the course of infection without producing a complete immunity could cause such a marked change in the effective chemotherapeutic dosage, it seemed of interest to compare several known strains of mice   Four standard pure-line strains were used Although the difference in susceptibility was again evidenced only by the time necessary to produce death, a marked difference existed in the amount of penicil lin necessary to effect protection

DISCUSSION

Impure penicillin is a more effective chemotherapeutic agent than crystal line penicillin G against at least four microorganisms belonging to different species   It is probable that such a difference may be demonstrable against a wide variety of organisms

Dunham and Rake (1945), working with *Treponema pallidum*, first suggested that impure penicillin may possess greater chemotherapeutic activity than crystalline penicillin G

The differences in the action of the pure penicillins in contrast to prepara tions of impure penicillin was first discussed in detail in a recent communi cation from this laboratory (Hobby *et al* , 1946)   Impure penicillin was shown to be 3 to 5 times more effective against hemolytic streptococcal infections in mice than crystalline penicillin G   Indeed, such impure penicillin was more effective than any of the five forms of purified penicillin with the exception of penicillin X

In the present report the same difference in activity has been demonstrated against pneumococcal infections in mice   Welch, Randall, and Price (1947) likewise have demonstrated recently a similar effect against infections due to *Eberthella typhosa*, and Rake, Dunham, and Donovick (1947) have confirmed their original observations on *Treponema pallidum* and have extended them to include the action of impure and pure penicillins on pox viruses grown in the chick embryo

The substance responsible for the greater activity of the impure penicillins in the streptococcal and pneumococcal infections is present in the original fer mentation liquors and may be recovered, during the purification of penicillin, in those fractions in which penicillin G is recovered   It does not crystallize with penicillin G, however   It is relatively heat-stable   Preliminary studies suggest that it is readily dialyzable   Only small amounts are necessary to en hance the activity of crystalline penicillin G   As indicated by the data of Welch *et al* , and also by ours, it is effective on penicillins X, dihydro-F, and K as well as on penicillin G

It has been shown previously (Hobby *et al* , 1946) that there is no correla tion between the presence of this factor and the source of the penicillin, its

potency in terms of units per mg, or its composition as evidenced by the *Bacillus subtilis, Staphylococcus aureus* differential ratio   These facts have been amply confirmed by Welch and his associates (1947)

That the more common degradation products probably are not in themselves responsible for enhancing the activity of crystalline penicillin G is suggested by the fact that enzyme- or alkali-inactivated crystalline G has failed to enhance active crystalline penicillin G when mixed with it   Likewise, no correlation exists between the presence, in impure penicillins, of the substance which enhances chemotherapeutic activity and the amount of phenylacetic acid present   A preparation of crystalline G, high in penicillinic acid, has shown no greater chemotherapeutic efficiency than crystalline penicillin G itself

That the factor which enhances the action of crystalline penicillin G in streptococcal and pneumococcal infections is not specifically correlated with the penicillin impurities only is suggested by the data presented in this communication   Certain apparently dissimilar agents may produce this effect   The penicillinase used for inactivation of impure penicillin, as well as the substances normally present in impure penicillin, have the ability to increase the efficacy of crystalline penicillin G   The penicillinase used in these studies was prepared from clarase   That the effect is not due to the penicillinase itself is indicated by the fact that a preparation of clarase, containing no penicillinase, was also highly effective, whereas a preparation of penicillinase from another source possessed little or no activity   Preliminary experiments indicate, furthermore, that certain mixtures of penicillins are more effective than crystalline G alone and that crystalline penicillin K may enhance the activity of crystalline G

Whether these enhancing substances act directly on the crystalline penicillin or whether they produce their effect through an alteration of some mechanism within the body is not known   That the latter is true, at least in part, is suggested by preliminary experiments in which inactivated impure penicillin was administered simultaneously with crystalline penicillin G but at different sites within the body

Tompsett, Schultz, and McDermott (1947) have recently demonstrated differences in the ability of the various penicillins to be bound by the albumin component of serum   Whereas only about 50 per cent of penicillin G is bound, as much as 90 per cent of penicillin K may be bound   Clowes and Keltch (1946) have demonstrated, furthermore, that larger amounts of penicillin K than G are removed from solution when exposed in a Warburg apparatus to the action of various amounts of muscle or liver slices   The effect of penicillin impurities or of penicillin K on the binding action of crystalline penicillin G is not known

The possible existence of an antibacterial agent effective *in vivo*, but not *in vitro*, must be considered   Esters of penicillin showing such activity were described by Meyer, Hobby, and Dawson (1943)   More recently Ramon, Richou, and Ramon (1946) have described a substance present in crude penicillins that possesses "antidotal" properties, and Miller and Boor (1947) have described protective action against certain bacterial endotoxins   Since the organisms used throughout this study elaborate a number of toxins, it is con-

ceivable that a substance capable of neutralizing one or more of these might enhance the protective action of crystalline penicillin

The data presented herein are preliminary in nature   Neither the nature of the enhancing substance present in impure penicillin nor the mechanism by which it acts has been determined.   That one or more such substances do exist seems undoubtedly true   That a variety of dissimilar substances, including ones not specific for impure penicillins may similarly enhance the action of crystalline penicillin G in streptococcal and pneumococcal infections is suggested   It seems possible that the factor (or factors) responsible for this form of enhancement may differ in nature or in their mechanism of action from those responsible for the enhancement of the action of penicillin on sarcoma cells, vaccinia virus   or bacterial toxins

The individual experiments described represent, in many instances only small numbers of animals and, therefore are probably not biostatistically valid   The total study however, represents over 25,000 animals and indicate a definite trend

Differences in host susceptibility may alter the amount of penicillin necessary to protect against certain infections   The relative efficiency of the various penicillins is therefore more significant than the actual dosage necessary to protect   An exact comparison of the chemotherapeutic value of various agents in animals is probably possible only in pure-line strains of relatively constant susceptibility

<center>CONCLUSIONS</center>

As previously reported   impure penicillin is 3 to 5 times more effective that crystalline penicillin G in protecting animals against experimentally produced hemolytic streptococcal infections in mice   The presence of this factor is not correlated with the potency in units per mg or with the *Bacillus subtilis, Staphylococcus aureus* differential ratio   Likewise it is not correlated with the amount of phenylacetic acid present   There is no evidence that it is associated with the degradation products of penicillin

As in the case of hemolytic streptococcal infections, impure penicillin is more effective than crystalline penicillin G in protecting animals against experimentally produced pneumococcal infections in mice   On a unitage basis (CD_{100}) the relative chemotherapeutic efficacy of penicillins X, dihydro-F, F, G, and K against this infection, under the experimental conditions used, was on the order of 302 180, 116 100 and 63, respectively, on a gravimetric basis, 194, 180, 107, 100 and 88, respectively

The factor in impure penicillin which enhances the chemotherapeutic activity of crystalline penicillin G is present in the original penicillin fermentation liquor and may be demonstrated during the extraction of penicillin in those fractions in which penicillin G is recovered

The chemotherapeutic activity of crystalline penicillin G may be enhanced by dissimilar substances not specific for impure penicillin   Furthermore, crystalline penicillin K may enhance the activity of crystalline penicillin G   The

effect of penicillin G plus K is greater than would be anticipated from the total
number of units present

The chemotherapeutic activity of penicillins X, dihydro-F, and at times K
may also be enhanced by penicillin impurities

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BACTERIAL DISPERSION BY SONIC ENERGY

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When a suspension of dispersed bacteria is subjected to intense sonic energy, sufficient to cause cellular rupture, there is a progressive decrease in the turbidity of the liquid with continued time of exposure. This change in turbidity can be used as a method of following the cellular disruption in that, when the proper wave length of light is employed, there is a direct relationship between the concentration of organisms and the absorption coefficient, or the transmittancy. This relationship is valid if the organisms are well dispersed. When they exist in aggregates, the extent of the reduction of the light passing through the liquid in which they are suspended will be somewhat less than if the same number of individuals were uniformly dispersed throughout the suspending medium.

In connection with some exploratory work, which was being done on the effect of sonic energy on various bacteria, in which a turbidimetric method of evaluating bacterioclasis was utilized, it was observed that, when the organisms under exposure existed in aggregates, the first detectable optical change was an *increase* in turbidity. Microscopically, it was confirmed that this increase was correlated with the extent to which aggregates were being separated.

If this dispersion effect could be utilized, it might well prove of value in connection with experimental procedures, such as nutrition studies, which are based on an assumption that such colonies which subsequently develop have been derived from single individuals.

It was for the purpose of preparing viable suspensions of well-dispersed organisms which normally exist in aggregates, such as clusters or chains, that the experiments to be described were undertaken.

METHODS AND EQUIPMENT

A laboratory model of a device, made by the Raytheon Manufacturing Company of Waltham, Massachusetts, for the application of intense sound energy to small quantities of liquids was utilized. It consists of a stainless steel cup, the bottom of which is a diaphragm that is vibrated by a laminated nickel structure connected to it. This magnetostrictor transducer is driven by an electronic power oscillator having an output of approximately 60 watts, at a frequency of about 9,000 cycles per second.

Absorption measurements were made by means of a balanced cell type of photoelectric colorimeter the light source of which had been corrected by means of filters to have its principal transmission at approximately 6,500 angstroms. The absorption coefficients which are plotted as ordinates on the accompanying

graphs represent the difference between the natural logarithm of the instrumen
tal scale readings when no organisms are present in the suspending liquid and
the natural logarithms of the instrumental scale readings under various experi
mental conditions when organisms are present

The conditions in which organisms existed, whether singly or in aggregates,
was also determined microscopically

<div align="center">EXPERIMENTAL RESULTS</div>

A small quantity, usually 20 ml, of a 24-hour culture of the organisms under
study was placed in the cup of the transducer, and subjected to intense sonic
energy for varying lengths of time   Prior to treatment the absorption coeffi

FIG 1 UNTREATED STAPHYLOCOCCUS ALBUS CULTURE

cient of the suspension was determined turbidimetrically, against sterile culture
medium as a blank   At suitable intervals during the course of the exposure to
sound, turbidity measurements were made   Thus the change in turbidity with
time of treatment could be plotted, and an optimum time for best dispersion
empirically established   It usually coincided with that point of the curve cor
responding to maximum turbidity   As stated before, the ordinates on the ac
companying graphs represent differences between the natural logarithms of
scale readings and are not absolute values   They are a function of the scale of
a particular instrument, and the dimension and shape of the cells in which the
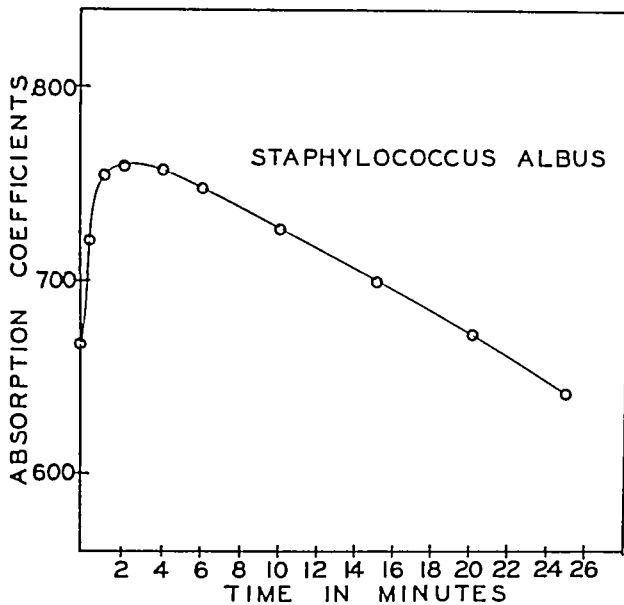liquids were held while turbidities were measured   However, the relationships

are relative and similar measurements made on any instrument will give comparable curves
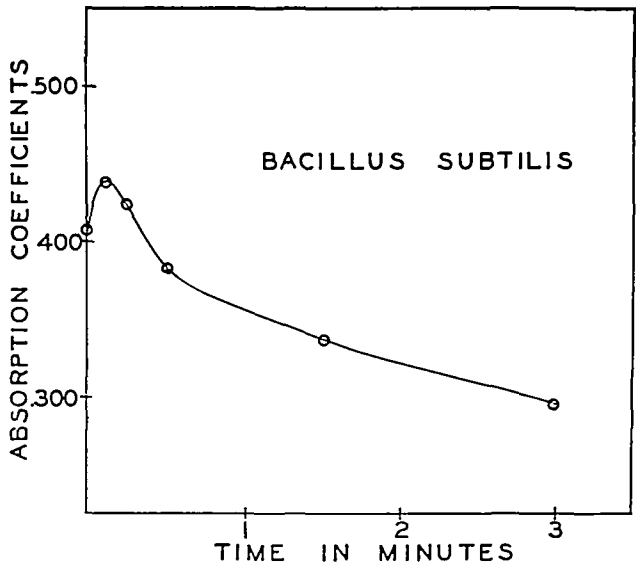
FIG 4 TURBIDITY CHANGES IN BACILLUS SUBTILIS SUSPENSION UNDER SONIC ENERGY
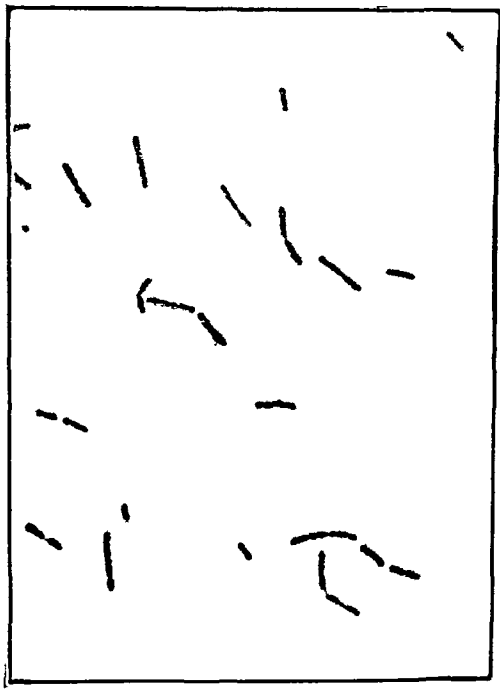
FIG 5 BACILLUS SUBTILIS SUSPENSION BEFORE TREATMENT

In order to avoid what would be rather pointless repetition, only three di persions of organisms will be reported in detail   Out of many cultures which
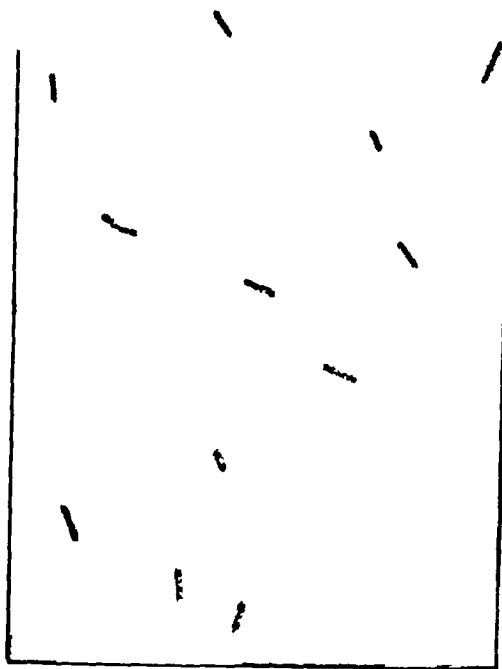
FIG 6 BACILLUS SUBTILIS DISPERSION AFTER SONIC EXPOSURE

HEMOLYTIC STREPTOCOCCUS
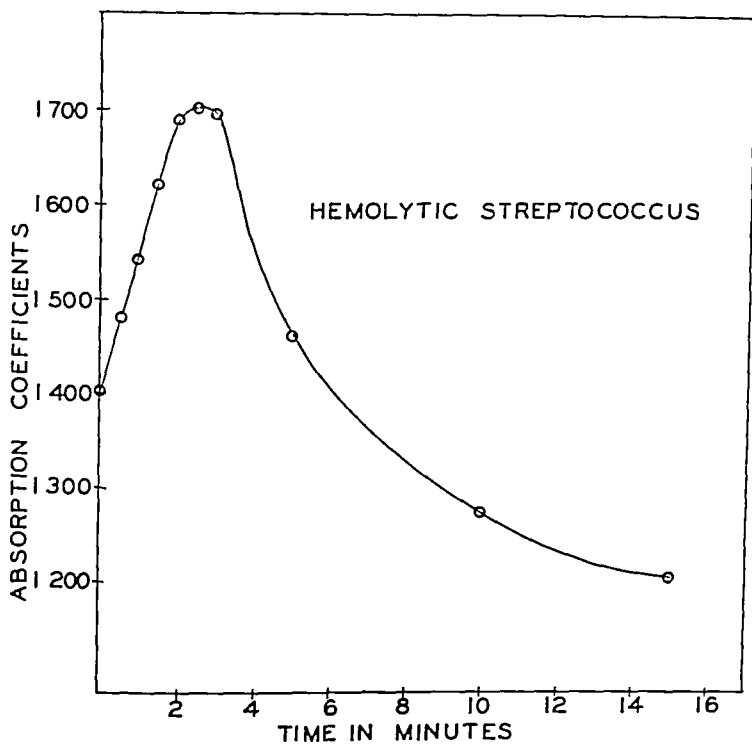
FIG 7 TURBIDITY CHANGES IN HEMOLYTIC STREPTOCOCCUS UNDER SONIC ENERGY

FIG 8 STREPTOCOCCUS SUSPENSION BEFORE TREATMENT

FIG 9 STREPTOCOCCUS DISPERSION AFTER SONIC EXPOSURE

were studied these three were selected as characteristic of types of aggregates which have been dispersed

Staphylococcus albus A 24-hour culture of *Staphylococcus albus* was grown in nutrient broth and its turbidity determined   A slide prepared at this time was photographed and is shown in figure 1   As can be seen, there were numerous aggregates   A 20-ml portion of the culture was placed in the cup of the transducer and exposed to intense sound energy at a frequency of alm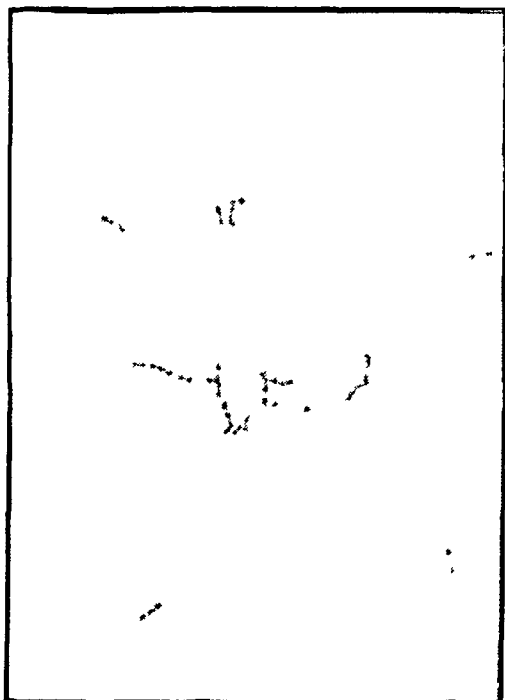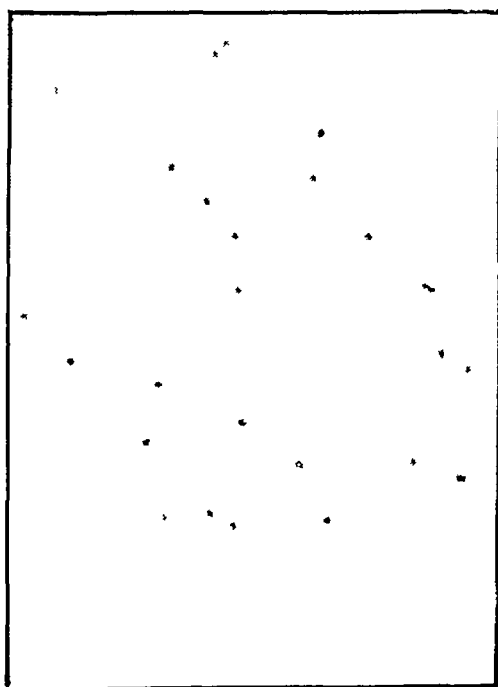ost 9,000 cycles per second   At selected intervals of time the turbidity of the liquid under treatment was determined   These turbidities expressed as absorption coefficients are shown in figure 2   It will be noted that there is a definite increase in turbidity up to 3 minutes, after which it decreases   This decrease in turbidity represents cell rupture, and is, in the case of aggregated organisms, an effect which follows their dispersion

The microscopic appearance of the same culture shown in figure 1 but after 20 minutes' exposure to sonic energy is shown in figure 3   It will be noted that the individual organisms are well separated

Bacillus subtilis A 24-hour culture of *Bacillus subtilis*, grown in nutrient broth, was treated under similar conditions to the *Staphylococcus albus* described in the foregoing experiment   Absorption coefficients obtained turbidimetrically are shown in figure 4   Figure 5 shows the appearance of the organisms before treatment, and figure 6 shows their appearance after 3 minutes of sonic exposure

Hemolytic streptococcus, group B A 24-hour culture of *Hemolytic streptococcus*, group B, grown in nutrient broth was exposed to sonic energy, and was followed both turbidimetrically and microscopically   Figure 7 shows the change in turbidity with continued time of treatment, figure 8 shows the culture as it appeared before treatment, and figure 9 is its appearance after 10 minutes of sonic exposure

SUMMARY AND CONCLUSIONS

Turbidimetric changes in three cultures exposed to sonic energy have been described and the dispersion of aggregates illustrated

A method is suggested for the preparation of well-dispersed suspensions of viable organisms which normally have a tendency to form aggregates

The exposure of cultures to sonic energy for suitable selected lengths of time provides a means for the separation of bacterial aggregates and the preparation of dispersed individual organisms

A MOUSE PROTECTION METHOD FOR THE ESTIMATION OF ANTIGENIC PNEUMOCOCCAL POLYSACCHARIDE IN SOLUTION

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In a study involving the preparation and testing of pneumococcus capsular polysaccharide, the need for a rapid method of assay of preparations of unknown antigenic potency became evident   Most bacterial vaccines are standardized on the basis of cell counts or total nitrogen determinations   Since such vaccines usually contain somatic protein as the principal antigen, determination of the number of cells or of the quantity of nitrogen is often a reliable criterion of antigenicity   Obviously, neither procedure can be used for the standardization of capsular carbohydrate preparations

Heidelberger and Kendall (1932) developed a method for the quantitative estimation of specific carbohydrate in solution which is based on the precipitation of this material by homologous type-specific antiserum   By this method, they were able to determine as little as 0 01 mg of type III pneumococcus polysaccharide   The procedure is quite satisfactory for many purposes, but it does not necessarily measure the antigenicity of the substance under test   For example, Felton (1934) found that heating type I pneumococcus polysaccharide in acid solution destroyed from 50 to 87 per cent of its immunizing activity, although the precipitin titer was not altered   Avery and Goebel (1933), working with type I pneumococcal capsular polysaccharide (subsequently referred to as SI), found that the deacetylated product precipitated specific antibody from homologous antiserum but that it was not antigenic when tested in mice   It is evident in this case that the method based on the precipitin reaction is not a reliable index of antigenicity   The method of Heidelberger and Kendall is applicable only when the specific carbohydrate is obtainable in a state of purity   Even then, a given weight of the pure material may vary widely in antigenicity owing to the effect of different methods of purification

Sevag (1934) reported that mice treated with 0 0001 mg of SI survived 1,000 fatal doses of type I pneumococcus   Schiemann *et al* (1931), using highly purified SI, found that the minimum amount necessary to produce demonstrable active immunity when injected into white mice was 0 01 µg   Since mice are so responsive immunologically to antigenic SI, it seemed that a mouse protection method might offer an extremely sensitive means of evaluating the antigenicity of experimental preparations

EXPERIMENTAL

A sample of highly purified SI was obtained[2] and tested in white mice   Animals weighing 18 to 22 grams were injected intraperitoneally on 4 successive

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[1] The Wm S Merrell Co , Fellow
[2] Dr Michael Heidelberger, College of Physicians and Surgeons, Columbia University, kindly supplied this material

days using a daily dose of 0 25 ml of tenfold dilutions of the SI in saline Five days after the last immunizing dose, the mice were challenged by intraperitoneal injections using a type I pneumococcus culture The virulence of the test culture was such that 1 0 ml of a 10^{-9} dilution was regularly fatal to control animals within 60 hours Dilutions of 10^{-7}, 10^{-8}, and 10^{-9} were used in order to be certain that some animals would receive 100 or more MLD In this work, there has been no need to use the LD_{50} method

The data shown in table 1 indicate that the minimum quantity of SI affording complete protection is 0 01 μg Using this material as a standard of potency, various crude and partially purified preparations were assayed by determining the highest dilution of the unknown which gives corresponding protection against type I pneumococcus

The results of a representative test are shown in table 2 The material under test was a partially purified SI solution obtained from a broth culture It is evident that 1 5,000,000 is the highest dilution of the unknown affording mouse

TABLE 1

Determination of the minimal quantity of standard SI which provides immunity against D pneumoniae, type I

Number of mice surviving 72 hours after inoculation

IMMUNIZING DOSE OF STANDARD SI	DILUTION OF CHALLENGING CULTURE			
	10^{-7}	10^{-8}	10^{-9}	10^{-11}
None (control)		0/6	0/6	6/6
1 0 μg	6/6	6/6	6/6	
0 1 μg	6/6	6/6	6/6	
0 01 μg	6/6	6/6	6/6	
0 001 μg	0/6	0/6	2/6	

protection in this case Since this dilution contains a quantity of SI equal in antigenic activity to 0 01 μg of the standard, the quantity of active SI in the original solution can be calculated as 50 mg per ml

Both tables 1 and 2 illustrate the definite end point which has been obtained routinely by this method In table 1 it can be seen that whereas quantities of the standard SI from 1 0 μg to 0 01 μg are completely effective in protecting mice, 0 001 μg is ineffective The lowest concentration of SI affording complete protection is considered to be the end point No significance is attached to the average survival time for individual groups, even though mice receiving less than 0 01 μg occasionally appear to survive somewhat longer than control animals The necessity for statistical treatment of data is thus eliminated In order to obtain these definite end points, it is necessary to standardize the conditions of culture and the virulence of the organism used for challenging Mice of the proper weight from four sources have been used for these tests with completely consistent results

The results obtained in an attempt to apply this method for the estimation of

SI in body fluids are shown in table 3   A rabbit weighing 3 kilograms was in-
-jected intravenously using 25 mg of SI   One hour later the rabbit was bled

TABLE 2

*Determination of the minimal dose of unknown SI solution which provides immunity against
D pneumoniae, type I*

Number of mice surviving 72 hours after inoculation

	DILUTION OF CHALLENGING CULTURE			
	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Controls				
Saline		0/6	0/6	6/6
Standard SI, 0 01 μg	6/6	6/6	6/6	
Dilutions of unknown SI				
1 500,000	6/6	6/6	6/6	
1 5,000,000	5/6	6/6	6/6	
1 50,000,000	0/6	0/6	1/6	

TABLE 3

*Estimation of SI in rabbit serum by determining active immunity developed in mice in response
to injection of the serum*

Number of mice surviving 72 hours after inoculation

	DILUTION OF CHALLENGING CULTURE			
	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Controls				
Saline		0/6	0/6	6/6
Normal serum 1 10	0/6	0/6	1/6	
SI standard, 0 05* μg	6/6	6/6	6/6	
Dilution of serum† from rabbit injected with 25 mg SI				
1 10	6/6	6/6	6/6	
1 1,000	6/6	6/6	6/6	
1 10,000	4/4	4/4	4/4	
1 100,000	0/4	0/4	0/4	

* Diluent was normal serum 1 10
† Serum from blood obtained by cardiac puncture 1 hour following intravenous injection
of SI

from the heart   Basing the calculations on body weight, the serum obtained
should have contained approximately 200 μg per ml   Calculations based on the
results given in table 3 indicate that it contained more than 100 μg but less than
1,000 μg per ml   No attempt was made to determine the quantity more accu-

rately, though it could doubtless be done by using additional dilutions of the unknown   It appears that the presence of body fluids does not interfere with the test, although naturally occurring immune substances in serum must be considered

DISCUSSION

The usual mouse protection tests, both active and passive, emphasize the determination of the number of lethal doses which treated animals resist rather than the determination of the quantity of antigen required to produce significant immunity   The passive mouse protection test of immune serum is, nevertheless, an indirect means of determining the quantity of antibody present (Heidelberger, Sia, and Kendall, 1930)   The principle involved in active mouse protection tests is quite different in that the response of the mouse to the antigenic stimulus determines the amount of antibody formed   It would appear that the determination of the minimal quantity of an antigen which elicits active immunity may be more significant as a measure of antigenicity than the number of lethal doses of the test organism which the mouse will resist   The method presented accomplishes this objective and gives easily interpreted end points

In preliminary work it appears that the time necessary to complete such a test can be shortened considerably   In one experiment it was found that a single injection of 1 0 ml of the material under test produced the same result as did 4 consecutive daily injections of 0 25 ml   This would shorten the time required for a determination by 3 days   It may not be necessary to allow 5 days between immunization and challenging of mice   In another experiment comparable results were obtained after a 4-day waiting period   Since significant deaths occur within 72 hours, this schedule permits a test to be completed within 8 days

The method described has been limited in application to pneumococcal materials which are antigenic in mice and for which an acceptable comparison standard can be obtained   There is no reason to believe that it could not be applied to the evaluation of other antigenic materials of a similar nature   The choice of dosage schedule, waiting period, and the standard to be used is arbitrary and can be planned to suit individual needs

SUMMARY

A mouse protection method for the estimation of antigenic pneumococcal polysaccharide in solution has been described

The principle of the test is based on the immune response of white mice to minute quantities of antigenically active polysaccharide

The procedure should be a useful supplement to methods based on the precipitin reaction because of its sensitivity and technical simplicity   Furthermore, the method described does not require standardization of antisera or purification of the antigen under test

This procedure provides a measure of antigenic potency rather than a measure of precipitable polysaccharide

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SIMULTANEOUS ADAPTATION A NEW TECHNIQUE FOR THE STUDY OF METABOLIC PATHWAYS

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During work on the oxidation of aromatic substances by *Pseudomonas fluorescens*, a useful technique for the elucidation of metabolic pathways by the analysis of adaptive behavior was discovered   Since it could undoubtedly be applied to many other microbial dissimilations, a brief account of its principles and applications seems merited

METHODS

Adaptation was determined manometrically, by following the oxygen uptake after addition of the substrate to a cell suspension in the Warburg apparatus All experiments were conducted at 30 C in an atmosphere of air, using 2 0 ml of cell suspension and 0 2 ml of 0 01 M substrate

One strain of *Pseudomonas fluorescens* (sti A 3 12) was used throughout The cells were grown on agar plates at 30 C and harvested after 20 to 45 hours by suspension in M/60 phosphate buffer (pH 7 0) After centrifugation they were resuspended in the same buffer mixture   The mineral media employed for cultivation of specifically adapted cells had the following composition specific carbon source, 0 1 to 0 25 per cent, NH_4NO_3, 0 1 per cent, K_2HPO_4, 0 1 per cent, $MgSO_4$, 0 05 per cent, and agar, 1 5 per cent, pH 7 0 to 7 2

The precision and sensitivity of the manometric technique make it ideal for studying adaptation to nonvolatile compounds, but complications arise when such substances as benzaldehyde are tested   Even in 0 01 M solution, the vapor pressure of benzaldehyde is sufficiently high at 30 C to cause a marked distillation from the side arm into the main compartment of the Warburg vessel, and adaptation consequently begins before the contents of the side arm are added to the cell suspension   Even when the period of thermal equilibration is held to a minimum, the effect is noticeable, showing up as an apparently more rapid adaptation to benzaldehyde than to nonvolatile substrates   Hence the results with this substance cannot be strictly compared to those obtained with the remaining aromatic compounds investigated

THEORY

If we accept the well-tested Kluyverian axiom (Kluyver, 1931) that every dissimilation is the result of a series of simple, chemically intelligible step-reactions, it follows that the complete oxidation of even a relatively small organic molecule will involve the formation of a large number of intermediate compounds In the case of microorganisms, the further probability exists that at least some

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of these intermediates will be attacked by adaptive enzymes   On the general theory of enzymatic adaptivity (cf Karström, 1937), cells adapted to attack the primary substrate should be adapted simultaneously to attack all the inter mediates formed during the oxidation of that substrate, but not to attack other substances the dissimilation of which is brought about by adaptive enzymes that fail to participate in the over-all dissimilatory process in question   Thus by growing cells on the primary substrate or on assumed intermediates and then testing for adaptation to a variety of related substances, one should be able to to obtain convincing evidence of whether or not assumed intermediates do actually occur, together (in positive instances) with information about their position in the reaction chain   The argument can be summarized in the follow ing three postulates

(1) If the dissimilation of a given substance A proceeds through a series of intermediates B, C, D, E, F, G   , and if the individual steps in this chain of reactions are under adaptive enzymatic control, then growth on a medium that contains A will produce cells that are simultaneously adapted to A, B, C, D, E, F, G,
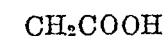
(2) If growth on A fails to adapt the cells to a postulated intermediate X, then X cannot be a member of the reaction chain

(3) Growth on E will adapt the cells for F, G,   but not necessarily for A, B, C, and D   The probability that growth on E will adapt the cells to precursors decreases with the number of intervening steps, i e , adaptation to D is more probable than adaptation to A

Postulate (3) perhaps requires a few additional words of explanation   In a complex dissimilation, it is conceivable that an enzyme will act at more than one stage in the dissimilatory process   Hence when two intermediates, say D and E, are separated by one enzymatic step, the possibility exists that the en zyme catalyzing that particular step (D→E) may also function later on in the oxidation of E, and that growth on E will also adapt the cells completely for the attack on D   However, if two intermediates, say B and E, are separated by several intervening steps (B→C→D→E), the probability that all three en zymes involved also take part in subsequent reactions is small, and thus growth on E is not likely to produce cells completely adapted for the oxidation of B

ANALYSIS OF A SPECIFIC BIOCHEMICAL PROBLEM BY MEANS OF SIMULTANEOUS ADAPTION

The application of the postulates may be illustrated with a relatively simple system, consisting of the following five compounds
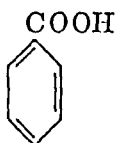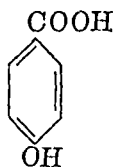
CH₂COOH   CHOHCOOH   CHO

phenylacetic   dl-mandelic   benzaldehyde
acid   acid

COOH

benzoic
acid

COOH

OH

para-hydroxybenzoic
acid

Each of them is readily utilized (in the case of the acids, as the sodium or potassium salt) by a strain of *Pseudomonas fluorescens* as the sole source of energy for aerobic growth in an otherwise mineral medium [2] Washed cell suspensions prepared from yeast extract agar are unadapted for the oxidation of these aromatic compounds the oxygen uptake remains at the autorespiratory rate for the first 40 to 70 minutes following the addition of the substrate, and then increases exponentially to a steady maximum rate which is maintained to the point of substrate exhaustion   Cells grown in the presence of any one of
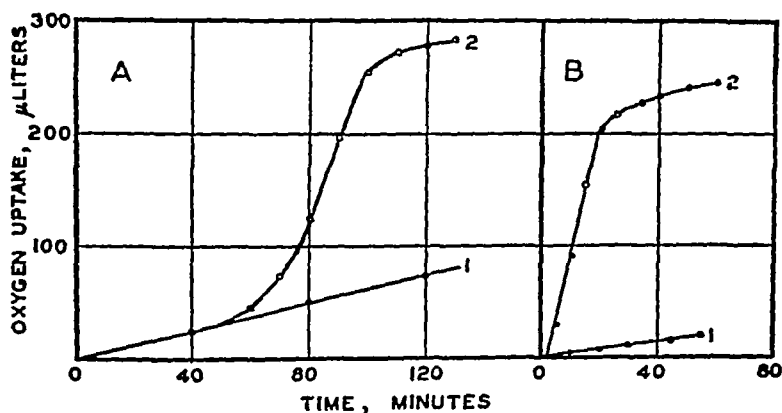
FIG 1 OXYGEN UPTAKE WITH 2 MICROMOLES OF BENZOATE BY PSEUDOMONAS FLUORESCENS GROWN ON YEAST EXTRACT AGAR (A) AND ON MINERAL BENZOATE AGAR (B)
1 = autorespiration, 2 = benzoate

the five substances show complete adaptation to that particular substance when tested in the same manner   These points are illustrated for benzoate in figure 1
It can be seen that the system is excellently suited for analysis along the lines of the postulates enunciated above, since it consists of five closely related compounds the oxidation of which by the biological agent employed is in all cases under primary adaptive control   Inspection of the structural formulae would suggest as a provisional hypothesis that these compounds comprise five successive members of an oxidative reaction chain

[2] Both isomers of mandelic acid are attacked at the same rate, and a racemic mixture has been used throughout the experiments herein reported

CH₂COOH   CHOHCOOH   CHO   COOH   COOH

→   →   →   →

OH
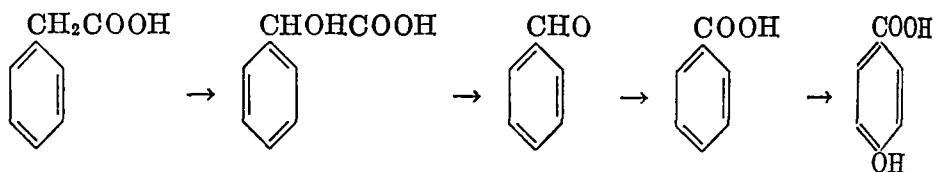
Analysis by simultaneous adaptation has provided conclusive evidence that this is not the case, and that in reality three separate primary oxidations are involved   The evidence for this is presented in figures 2, 3, 4, and 5   Cells

TIME, MINUTES

FIG 2 OXYGEN UPTAKE WITH 2 MICROMOLES OF VARIOUS AROMATIC COMPOUNDS BY PSEUDOMONAS FLUORESCENS GROWN ON MINERAL BENZOATE AGAR
1 = autorespiration, 2 = benzoate, 3 = p-hydroxybenzoate, 4 = mandelate, 5 = phenyl acetate

were grown on four mineral agar preparations containing, respectively, benzoate, para-hydroxybenzoate, mandelate, and phenylacetate and then tested manometrically for adaptation to the four acids and to benzaldehyde   Only the data for the four acids are shown on the graphs

Figure 2 demonstrates that para-hydroxybenzoate is not an intermediate in

the oxidation of benzoate, since benzoate-grown cells are unadapted for its oxidation The immediate attack at maximum rate on *para*-hydroxybenzoate by cells grown in its presence (figure 3) shows that the initial lag in its oxidation by benzoate-grown cells cannot be ascribed to permeability effects
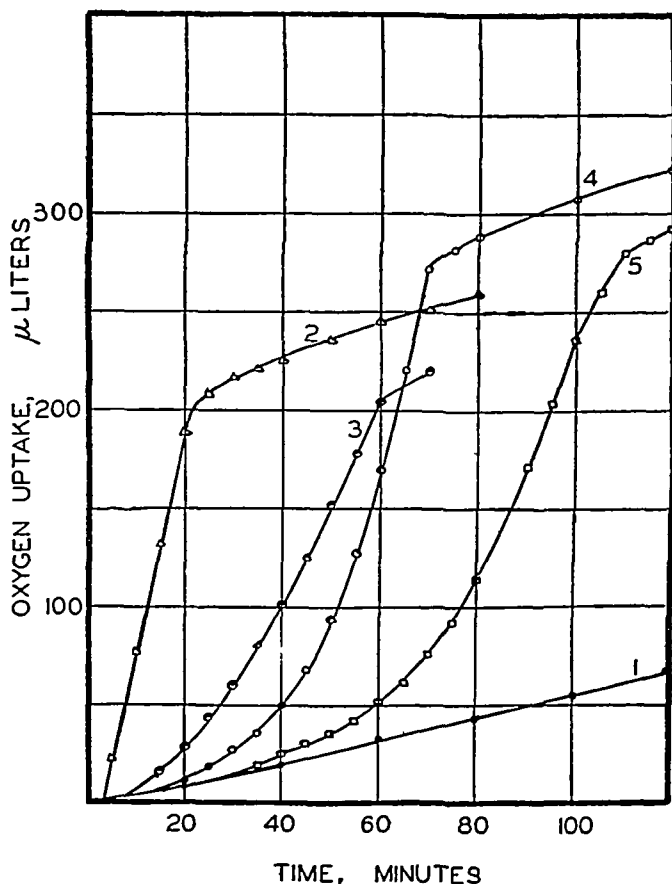

FIG 3 OXYGEN UPTAKE WITH 2 MICROMOLES OF VARIOUS AROMATIC COMPOUNDS BY PSEUDOMONAS FLUORESCENS GROWN ON MINERAL *p*-HYDROXYBENZOATE AGAR
1 = autorespiration, 2 = benzoate, 3 = *p*-hydroxybenzoate, 4 = mandelate, 5 = phenylacetate

Figure 4 shows that benzoate is oxidized at the same rate as mandelate by cells grown on the latter substrate, suggesting that benzoate is an intermediate in mandelate oxidation As might be expected if this were the case, mandelate-grown cells are unadapted to *para*-hydroxybenzoate

The results presented in figure 5 for cells grown on phenylacetate are perhaps the most interesting of all In the first place, the typically adaptive curve for the oxidation of benzoate proves that phenylacetate cannot be oxidized along this pathway The curve for mandelate shows a new feature it has a double break, the initial rapid rise in oxygen uptake being followed (after a brief re-

turn to the autorespiratory rate) by an exponential rise that parallels with rea
sonable closeness, but at the higher absolute level initially established, the
strictly adaptive curve for benzoate The first break comes at a point that
corresponds approximately to an oxygen uptake of one mole per mole of sub-
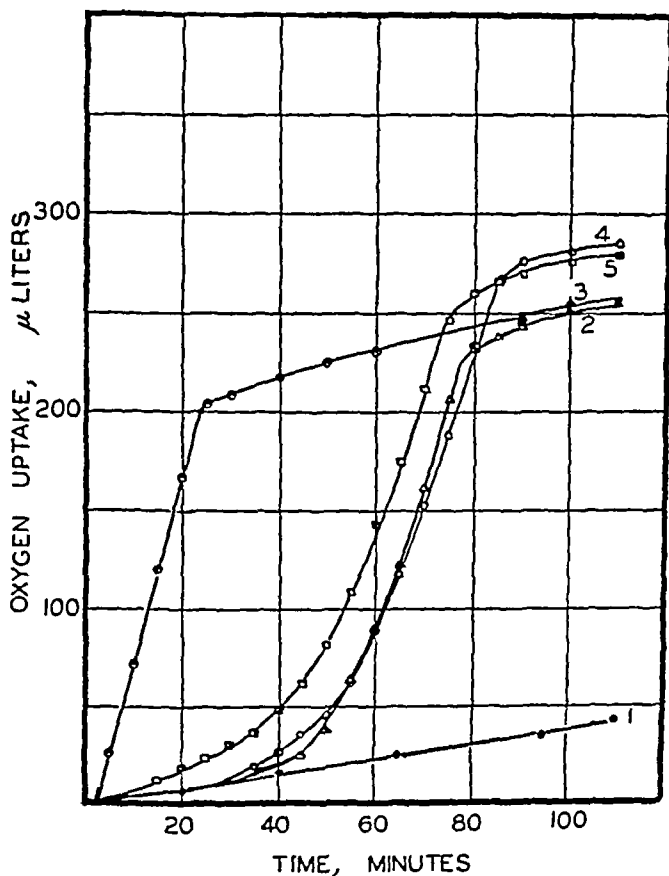strate The only likely interpretation of such a curve is that growth on phenyl

FIG 4 OXYGEN UPTAKE WITH 2 MICROMOLES OF VARIOUS AROMATIC COMPOUNDS
BY PSEUDOMONAS FLUORESCENS GROWN ON MINERAL MANDELATE AGAR
1 = autorespiration, 2 = benzoate, 3 = p-hydroxybenzoate, 4 = mandelate, 5 = phenyl
acetate

acetate has activated the dehydrogenases involved in the initial oxidation of
mandelate to benzoate—

$$\text{CHOHCOOH} \bigcirc + H_2O \rightarrow \text{COOH} \bigcirc + CO_2 + 4H$$

—but not (as shown also by the curve for benzoate) the enzyme systems oper
ating at later stages

The peculiar action of phenylacetate-grown cells on mandelate made possible a further experiment in substantiation of the hypothesis that benzoate really is an intermediate in the oxidation of mandelate   Adaptation to either benzoate or phenylacetate singly fails to bring about complete adaptation to mandelate (figures 2 and 5), but if the deductions drawn from the experiments above are correct, cells adapted to *both* of these substances should also be adapted, by a
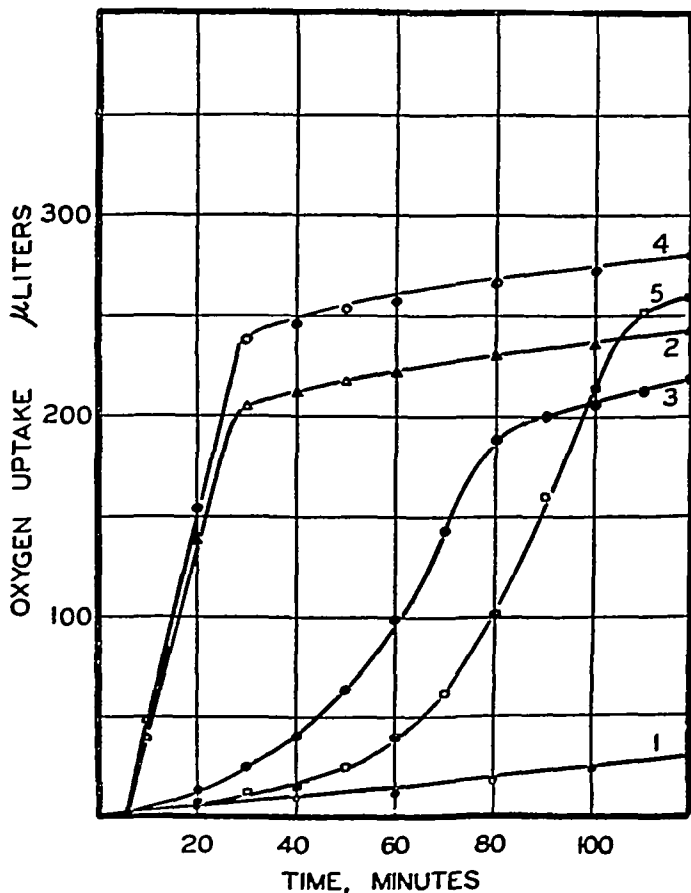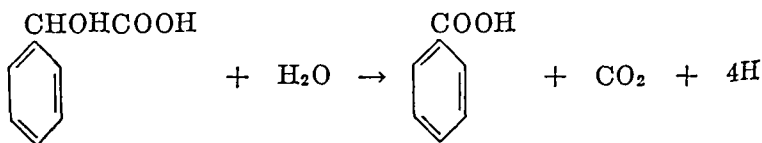

FIG 5 OXYGEN UPTAKE WITH 2 MICROMOLES OF VARIOUS AROMATIC COMPOUNDS BY PSEUDOMONAS FLUORESCENS GROWN ON MINERAL PHENYLACETATE AGAR
1 = autorespiration, 2 = benzoate, 3 = *p*-hydroxybenzoate, 4 = mandelate, 5 = phenyl-acetate

process of complementary activation, to mandelate   As shown in figure 6, this expectation is realized

The data with benzaldehyde indicate that this substance is probably an intermediate in the oxidation of mandelate to benzoate, although for the reasons mentioned earlier the results are not so clear-cut as those with the aromatic acids   Mandelate-grown cells are completely adapted to benzaldehyde, and phenylacetate-grown cells show "semiadaptation" of the same sort as that discussed above for mandelate, with the difference that the first break in the curve for

benzaldehyde oxidation comes at a level of about 0 5 moles of oxygen per mole of substrate, in accordance with the equation

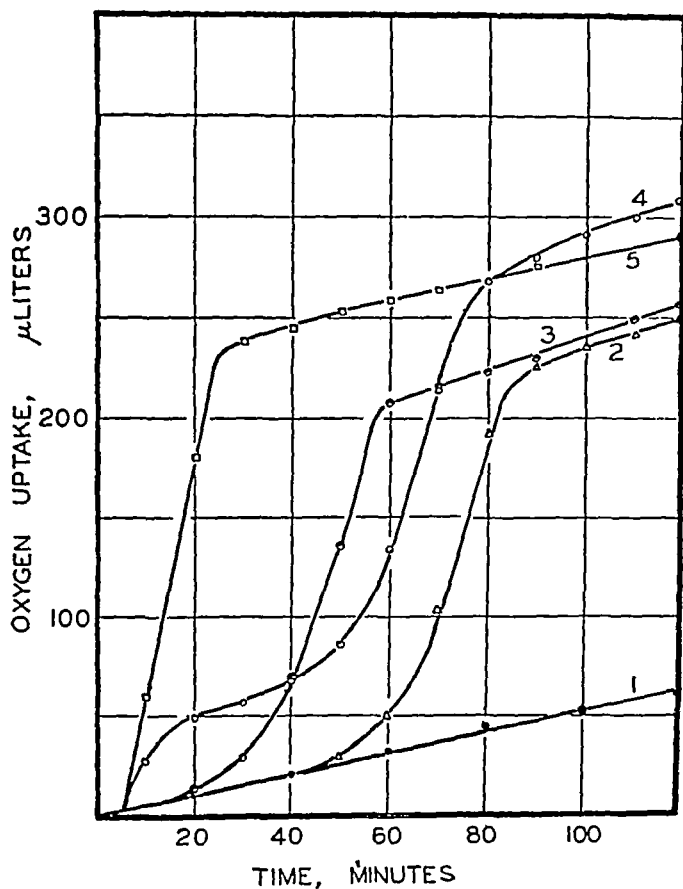CHO   COOH

$$\text{CHO} + H_2O \rightarrow \text{COOH} + 2H$$
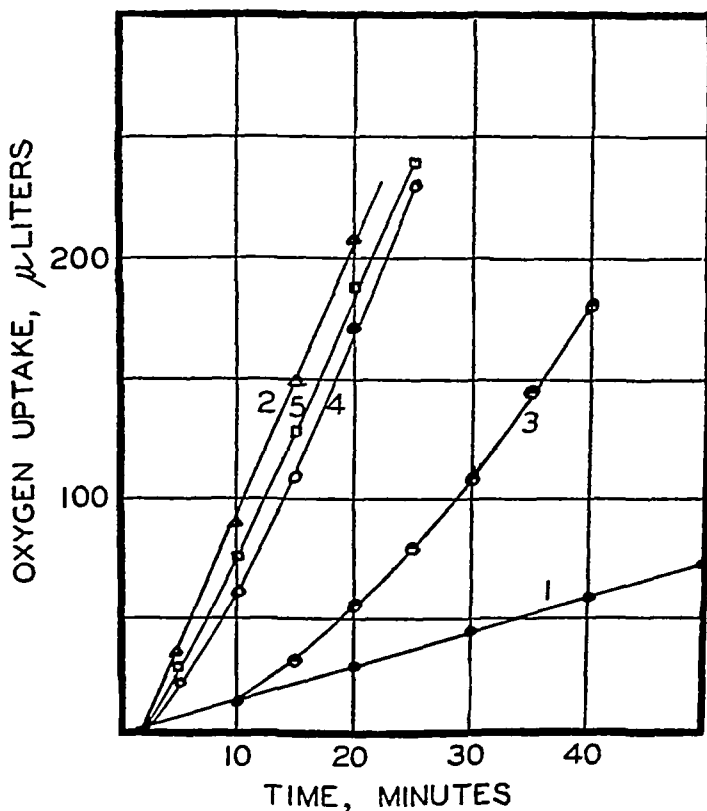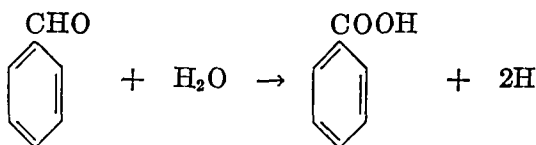
FIG 6 OXYGEN UPTAKE WITH 2 MICROMOLES OF VARIOUS AROMATIC COMPOUNDS BY PSEUDOMONAS FLUORESCENS GROWN ON MINERAL BENZOATE AGAR AND "PREADAPTED" TO PHENYLACETATE BY INCUBATION IN THE PRESENCE OF THIS SUBSTANCE FOR 60 MINUTES PRIOR TO THE EXPERIMENT
1 = autorespiration, 2 = benzoate, 3 = p-hydroxybenzoate, 4 = mandelate, 5 = phenyl acetate

Interestingly enough, benzoate-grown cells show complete adaptation for benzaldehyde, suggesting that the benzaldehyde dehydrogenase also functions in the later stages of benzoate oxidation   The adaptation of benzoate-grown cells to benzaldehyde but not to mandelate is a good illustration of the third postulate
    The net result of these experiments has been to show the existence in *P fluorescens* of three separate oxidative mechanisms involving aromatic substances

CHOHCOOH   CHO   COOH

→   →   → ?

CH₂COOH

→ ?

COOH

→ ?

OH

$$\text{CHOHCOOH} \rightarrow \text{CHO} \rightarrow \text{COOH} \rightarrow ?$$

$$\text{CH}_2\text{COOH} \rightarrow ?$$

$$\text{COOH, OH} \rightarrow ?$$
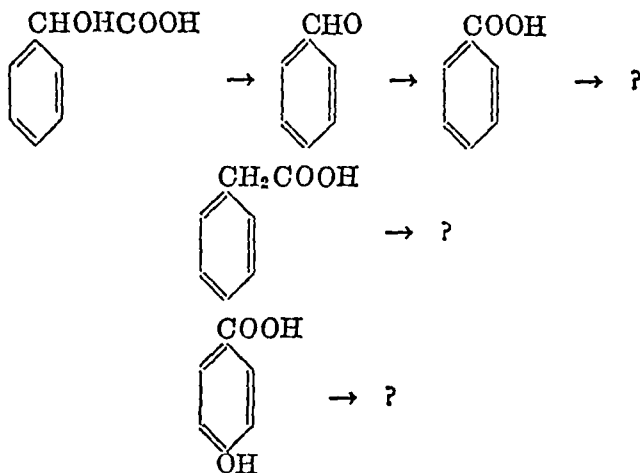
This information could not have been obtained from data on utilization, or even from data on absolute rates of oxidation, which are quite similar for all five compounds

The fact that growth on phenylacetate activates the dehydrogenases involved in the oxidation of mandelate and benzaldehyde to benzoate probably indicates that these two enzymes are nonspecific, and also function at some stage in the oxidation of phenylacetate   Lack of enzymatic specificity is, of course, a limitation to the validity of the technique, and necessitates judicious evaluation of positive findings   It seems most improbable, however, that exactly the same set of enzymes would be involved in two different complex oxidative processes, so that even if growth on one substance activates nonspecifically the first step or steps in the oxidation of another substance, lack of adaptation at some later point in the chain of events will temporarily halt the attack, resulting in a "semiadapted" curve for oxygen uptake   Indeed, the occurrence of such behavior should in itself provide valuable information as to the course of the reaction   It is difficult to see how clear lack of adaptation to a postulated intermediate can be regarded as anything but conclusive negative evidence, provided that permeability effects have been ruled out by a demonstration that cells adapted to the substance in question can oxidize it immediately at the maximum rate

One further point, at present highly speculative, deserves brief mention It does not seem excluded that *relative rates* of adaptation may also provide indications of biochemical interrelationships   A case in point is the relatively rapid adaptation of cells grown either on benzoate or on phenylacetate to *para*-hydroxybenzoate (figures 2 and 5)   A possible interpretation of this behavior is that all three substances have a common intermediate, from which *para*-hydroxybenzoate is separated by fewer steps than either of the other two, with the consequence that cells grown on benzoate or phenylacetate need to produce fewer adaptive enzymes for the attack on *para*-hydroxybenzoate than for the attack on one another

The systematic use of simultaneous adaptation, coupled with the other kinds of data obtainable from manometric experiments, should be particularly valuable in the study of those dissimilatory processes that have so far proved least amenable to analysis—namely, rapid and complete oxidations of relatively complex substances The only prerequisite is that the enzymatic repertoire of the biological agent employed should be largely adaptive

SUMMARY

The theory of simultaneous adaptation as a method for the analysis of metabolic pathways is described, and its application is illustrated by a specific example the oxidation of five aromatic compounds by a strain of *Pseudomonas fluorescens*

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SEROLOGICAL STUDIES OF THE GENUS XANTHOMONAS

III The Xanthomonas vascularum and Xanthomonas phaseoli Groups, the Intermediate Position of Xanthomonas campestris

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Previous studies (Elrod and Braun, 1947a) indicated that the genus *Xanthomonas* showed evidence of antigenic divisibility   Since the individual species of the genus cannot easily be differentiated by the physiological determinative procedures ordinarily used in bacteriology, it was suggested that serological information might be utilized as an aid in the classification of these organisms   Further study by means of agglutinin absorption in one of the divisions, arbitrarily designated as the *X translucens* group (Elrod and Braun, 1947b), argued against the possibility that all described species within this group represented distinct serological entities   Nevertheless, it was possible to define certain specific immunological components within this group which apparently were correlated with host specificity   Discrepancies in this respect were indicated by the serological identity of organisms isolated from such widely separated sources as pumpkin, begonia, and certain grasses

The present paper deals with a continuation of serological study of three other immunological divisions of the genus *Xanthomonas*—the *X vascularum*, *X phaseoli*, and *X campestris* groups   It was previously observed (Elrod and Braun, 1947a) that, by using heavily mucoid cultures, a relationship could be shown to exist between the *"vascularum"* and *"phaseoli"* groups   The *X vascularum* type organism agglutinated in the *X phaseoli* group antiserums, but the reciprocal reaction was not observed   It was found that, by freeing the cultures of the mucoid material, this unilateral relationship was lost, there were no agglutination reactions between the two groups   The two organisms in the *X campestris* group, *X campestris* and *X barbareae*, tended to link the two groups in question   All of the cultures in the *X vascularum* and *X phaseoli* groups agglutinated in *X campestris* and *X barbareae* antiserums   The latter two organisms reacted in all of the individual antiserums of the two larger groups   In the present investigation the nature of this intermediate role was investigated, and the members of the *"vascularum"* and *"phaseoli"* groups were compared by means of agglutinin absorption

ORGANISMS OF THE XANTHOMONAS VASCULARUM, XANTHOMONAS PHASEOLI, AND XANTHOMONAS CAMPESTRIS GROUPS

The species and subspecies that compose these three groups have a wide and varied host range   Table 1 presents information pertaining to natural hosts,

isolates employed in the study, and the original authority for the species in question   It is to be noted that the susceptible hosts extend through many genera and families of the higher plants   *Xanthomonas vascularum* is apparently specific for sugar cane, whereas *X vesicatoria* has a known host range limited to certain of the *Solanaceae*, more particularly pepper and tomato   *X vesicatoria* v *raphani* is infective for pepper and tomato as well as for the radish   In this

TABLE 1

Species and subspecies with representative hosts in the Xanthomonas vascularum, Xanthomonas phaseoli, and Xanthomonas campestris groups

SPECIES	ISOLATES STUDIED	NATURAL HOST OR HOSTS	AUTHORITY
X vascularum	XVI, XV46	Sugar cane (*Saccharum officinarum*)	Cobb, 1893
X vesicatoria	XV3 XV4, XV7, XV8, XV13, XV14	Tomato (*Lycopersicon* spp) Pepper (*Capsicum* spp)	Doidge 1921
X vesicatoria v *raphani*	XV16, XV16A	Radish (*Raphanus* spp) Tomato (*Lycopersicon* spp) Pepper (*Capsicum* spp)	White 1930
X papavericola	XP5 XP17	Poppy (*Papaver rhoeas*)	Bryan and McWhorter, 1930
X hederae	XH1, XH6	Ivy (*Hedera helix*)	Arnaud, 1920
X incanae	X13	Stock (*Mathiola incana*)	Kendrick and Baker, 1942
X taraxaci	XT11	Russian dandelion (*Taraxacum kok-saghyz*)	Niederhauser, 1943
X campestris	XC2, XC3, XC10, XC15	Cabbage, cauliflower (*Cruciferae* spp)	Pammel 1895
X campestris v *armoraciae*	XC4	Horse radish (*Armoraciae* spp)	McCulloch, 1929
X barbareae	XB1, XB2	Winter cress (*Barbareae vulgaris*)	Burkholder, 1941
X phaseoli	XP1 XP14, XP28	Beans (*Phaseolus* spp *Dolichos* spp *Lupinus* spp)	Smith, 1897
X phaseoli v *fuscans*	XP18 XP19, XP26	Beans (*Phaseolus* spp)	Burkholder, 1930
X geranii	XG1, XG1a, XG3, XG4	Geranium (*Geranium* spp)	Burkholder 1937
X pelargonii	XP7 XP8, XP15	House geranium (*Pelargonium* spp)	Brown 1923
X malvacearum	XM2, XM13, XM14	Cotton (*Gossypium* spp)	Smith, 1901

regard it is similar to *X barbareae*, *X campestris*, and *X campestris* v *armoraciae* in being pathogenic on some of the *Cruciferae*   The poppy is host to *X papavericola*, *X hederae* infects ivy, *X incanae*, stocks, *X taraxaci*, the Russian dandelion, and *X barbareae*, winter cress   *X geranii* and *X pelargonii* attack geraniums in different genera, *Geranium* and *Pelargonium*   Cotton is affected in world-wide distribution by *X malvacearum*   Beans of different genera are susceptible to *X phaseoli* and *X phaseoli* v *fuscans*   The latter owes its distinction to the elaboration of a brown pigment

Without recourse to repetition the reader is referred to table 1 and table 2, part A (Elrod and Braun, 1947a) for the cross-agglutination aspects of the three serological groups under consideration

Random sampling of absorbing combinations was not successful in producing specific serums in the *Xanthomonas vascularum* group   As a consequence, reciprocal absorptions were conducted between all eight organisms, with a few exceptions concerning *X incanae*   In no case did these mirror absorptions indicate that any two of these species were serologically identical   Nor was it possible to produce a specific antiserum for the homologous organisms by absorption with any one of the heterologous types   The patterns produced by these absorptions were varied, giving the impression that we were dealing with a multitude of group-specific factors   It was felt that the use of different organisms in absorbing combinations would produce specific serums   This laborious procedure was not deemed warranted   In table 2 are indicated typical results obtained when individual serums were absorbed with single cultures   On absorbing *X vesicatoria* (XV14) antiserum with *X vesicatoria* v *raphani* (XV16), all heterologous agglutinins except those for *X hederae* (XH1) were removed   When the foregoing antiserum was absorbed by the latter organism, the agglutinins for the absorbing culture and *X vascularum* (XV1) were obliterated   When *X papavericola* (XP5) was used to absorb this serum, all agglutinins remained except those for XP5 and *X incanae* (XI3)   The probable antigenic uniformity of *X vesicatoria* is indicated by the complete removal of antibody by XV7 or XV13, other isolates of this species   Dissimilar patterns are produced also by absorbing *X vascularum* (XV1) antiserum   Generally, reactive components remained for most of the antigens concerned when this serum was acted upon by *X hederae* (XH1), *X papavericola* (XP5), *X vesicatoria* v *raphani* (XV16), or *X campestris* v *armoraciae* (XC4)   The latter organism was found to have closer immunological affinities for the *X vascularum* group than for *X campestris*   Absorption of the antiserum for *X campestris* v *armoraciae* (XC4) by *X vascularum*, *X vesicatoria*, and *X papavericola* left sizable group components   Absorption, however, with *X vesicatoria* v *raphani* (XV16) removed all agglutinins, homologous and heterologous   Reciprocally, a large homologous-reacting fraction remained when the *raphani* variety antiserum was absorbed with *X campestris* v *armoraciae* (XC4)   This indicated that the two organisms differ only in additional components for *X vesicatoria* v *raphani*, not present in *X campestris* v *armoraciae*   This complete unilateral absorption has been observed in other combinations, i e , *X taraxaci* with *X vesicatoria* v *raphani*, *X papavericola* with *X vesicatoria*, and *X hederae* with *X vascularum*   In each instance the reciprocal absorption did not negate the homologous reaction

In contrast to the results obtained in the *Xanthomonas vascularum* group are those noted with *X phaseoli* group organisms   It is to be observed (table 3) that absorption of any antiserum of organisms in this category by any heterologous culture of the group leaves only species-specific factors   It can be assumed that the group-specific components are identical and uniform in distribution

TABLE 2

Absorption of Xanthomonas vascularum group antiserums by members of the group

ORGANISM AGGLUTINATED	XV14 ANTISERUM ABSORBED WITH					XV1 ANTISERUM ABSORBED WITH					XC4 ANTISERUM ABSORBED WITH				
	UNABSORBED	XV16	XV11	XP5	XV7 or XV13	UNABSORBED	XH1	XP5	XV16	XC4	UNABSORBED	XV1	XV14	XP5	XV16
X *vesicatoria* (XV11)	+++	++++	++++	++++	−	+++	+	++	++	+	++++	+++	−	++	−
X *vesicatoria* v *raphani* (XV16)	+++	+	++	+	−	+++	−	++	−	−	++++	++	−	−	−
X *hederae* (XH1)	+++	+	−	++	−	++++	−	+++	++	++	++++	−	−	−	−
X *incanae* (X13)	++++	−	+	−	−	++++	−	++	−	−	++++	−	−	−	−
X *papavericola* (XP5)	++++	−	++	−	−	++++	−	−	+	+	++++	+++	+++	+++	+
X *campestris* v *armoraciae* (XC4)	++++	−	++	+	−	++++	++	++	++	++	++++	+++	++	+++	+
Y *vascularum* (XV1)	+++	−	−	−	−	++++	++	++	++	++	++	−	−	−	−
X *taraxaci* (XT11)	++++	−	++	+	−	++++	−	++	++	−	++++	+	+	++	−

− = no agglutination at 1 50
+ = agglutination at 1 50 or 1 100
++ = agglutination at 1 200 or 1 400
+++ = agglutination at 1 800 or 1 1,000
++++ = agglutination at 1 3,200 or above

Of the five organisms in this division two were found to be seiologically identical They were X *gerani* and X *pelargoni* They reacted identically in all absorbing combinations and also were alike as shown by mirror absorption with their respective immune serums (table 3)

TABLE 3

Agglutinin absorption experiments in the Xanthomonas phaseoli group

SERUM	ABSORBED WITH	ORGANISM AGGLUTINATED WITH				
		XG4	XP7	XP14	XM13	XP19
X *gerani* (XG4)	—	++++*	++++	+++	+++	++
	XP7	—	—	—	—	—
	XP14	++	++	—	—	—
	XM13	+++	++	—	—	—
	XP19	++	++	—	—	—
X *pelargoni* (XP7)	—	++++	+++	+++	+++	++
	XG4	—	—	—	—	—
	XP14	+++	+++	—	—	—
	XM13	++	+++	—	—	—
	XP19	++	++	—	—	—
X *phaseoli* (XP14)	—	++++	++++	++++	++++	++
	XG4	—	—	++	—	—
	XP7	—	—	++	—	—
	XM13	—	—	+++	—	—
	XP19	—	—	++	—	—
X *malvacearum* (XM13)	—	++	+++	+++	++++	+++
	XG4	—	—	—	++	—
	XP7	—	—	—	++	—
	XP14	—	—	—	++	—
	XP19	—	—	—	+++	—
X *phaseoli* v *fuscans* (XP19)	—	+++	++	++	+++	++++
	XG4	—	—	—	—	+++
	XP14	—	—	—	—	++
	XM13	—	—	—	—	+++

, * See table 2 for explanation of symbols

The intermediate position of *Xanthomonas campestris* and X *barbareae* between the X *vascularum* and X *phaseoli* groups is unique It was evident from previous investigations (Elrod and Braun, 1947a), and as is indicated by table 4, that organisms of both groups reacted strongly in X *campestris* (XC10) and X *barbareae* (XB2) antiserums Also, these two species reacted in all of the individual serums of the two groups There was, however, no evidence of reaction between the groups per se, when mucoid-free antigens were used (*loc cit*)

The similarity of reaction manifest between *Xanthomonas campestris* and X *barbareae* led to the performing of mirror absorption tests between the two This

resulted in complete reduction of activity in each case, indicating the serologic identity of the two organisms

Absorption of *Xanthomonas campestris* (XC10) antiserum (table 4) by any member of the *X vascularum* division removed all of the agglutinins for the group, while reducing the components peculiar to *X campestris*, and not removing the factors active against the *X phaseoli* group Likewise, absorption of *X campestris* immune serum by members of the *phaseoli* group removed antibodie active for the latter organisms, but failed to obliterate activity for *X campestri* and the *vascularum* group Multiple absorption with *X geranii* (XG4) and *X*

TABLE 4

Absorption experiments of Xanthomonas campestris antiserum by Xanthomonas barbarea and Xanthomonas vascularum and Xanthomonas phaseoli group organisms

ORGANISM AGGLUTINATED	UNAB SORBED	ABSORBED BY							
		XV14	XV16	XH1	XC4 and XG4	XB2	XG3	XM13	XP14
X vesicatoria (XV14)	+++	—*	—	—	—	—	++	++	+
X vesicatoria v raphani (XV16)	++	—	—	—	—	—	++	++	++
X hederae (XH1)	++	—	—	—	—	—	++	+	++
X incanae (X13)	++	—	—	—	—	—	+	++	+
X papavericola (XP5)	++	—	—	—	—	—	+++	++	++
X campestris v armoraciae (XC4)	+++	—	—	—	—	—	++	++	++
X vascularum (XVI)	+++	—	—	—	—	—	++	++	++
X taraxaci (XT11)	+++	—	—	—	—	—	+++	++	+
X barbareae (XB2)	++++	+++	++	++	++	—	++	++	++
X campestris (XC10)	+++	+++	++	++	++	—	+++	++	++
X geranii (XG4)	+++	+++	++	+	—	—	—	—	—
X pelargonii (XP7)	+++	+++	++	+	—	—	—	—	—
X phaseoli (XP14)	++	+++	+	+	—	—	—	—	—
X malvacearum (XM13)	++	++	+	+	—	—	—	—	—
X phaseoli v fuscans (XP19)	++	++	+	+	—	—	—	—	—

* See footnote to table 2 for explanation of symbols

campestris v armoraciae (XC4) left *X campestris* (XC10) antiserum only specific agglutinins (table 4) The latter species is characterized by a factor common to the *phaseoli* group, one common to the *vascularum* group, in addition to species specific components

DISCUSSION

It seems apparent from the studies made in the *Xanthomonas transi* (Elrod and Braun, 1947b) and *X phaseoli* groups that specific antiserums many species of the genus *Xanthomonas* could easily be prepared Even organisms of the *X vascularum* group, specific serums should be available

proper absorbing combinations   The number of group-specific factors apparently vary considerably, and, likewise, their distribution would appear to produce a variety of serological patterns   It was felt unadvisable to expend the added labor on a complete antigenic analysis, or to prepare at this time specific antiserums for the *X vascularum* organisms

It appears certain, however, that many of the well-recognized *Xanthomonas* species (based on known host range) are distinct immunologically   Several questions arise in this regard   Is this serological specificity attributable to the prolonged growth of the organism on a well-defined host? Is the ability to infect only certain hosts due to an antigenic uniqueness?   Or finally, is there no correlation between the antigenic make-up of the bacterial cell and its ability to infect specific hosts?

It is interesting to conjecture that members of the genus *Xanthomonas* have evolved from a common organism present as a saprophyte either in the soil or on the external surfaces of plants   The assumption that the many xanthomonads have arisen from a common stock is not unreasonable in the light of the uniformity of physiological characteristics evident throughout the genus   At the same time the gradual (but sometimes abrupt) antigenic differences manifest between most of the recognized species lends credence to common ancestry   If a hypothetical ancestor is assumed for the genus, then it can be argued that through variation and selection of this primitive form an organism arose that was capable of invading and surviving in plant tissues, and that, through association with a host of definite structure, an antigenic mosaic peculiar to a specific organism evolved   On this assumption, a change in antigenic structure should take place along recognized lines if a species of *Xanthomonas* foreign to a given host was made to proliferate and thrive in that host   Also, if a change in antigenic structure were possible *in vitro* (similar to that recognized in the pneumococci) there should be an accompanying shift in specific host   Reid *et al* (1942) have, in fact, made the claim that *Pseudomonas fluorescens* changed antigenically to become identical with *Phytomonas tabaci* in the "M" phase by association on clover plants during a single season   These experiments are open to criticism, however, because of the lack of rigidly controlled experimental conditions

Not all xanthomonads are immunologically defined, assuming that all of the described species are valid   The serological identity of *Xanthomonas campestris* and *X barbareae* is not in the least illogical   The latter organism was isolated and described by Burkholder (1941) in search for a reservoir of *X campestris* The xanthomonad isolated by him was declared to be a different entity from *X campestris*   This was occasioned by the apparent inability of the two organisms to cross-infect their hosts of isolation   The description of the winter cress organism failed to distinguish it from *X campestris*   Serologically we found the two to be identical   Inasmuch, however, as winter cress (*Barbareae vulgaris*) belongs in the family *Cruciferae*, this antigenic similarity with *X campestris*, whose hosts number many *Cruciferae*, can be reconciled   Yet it is far more difficult to resolve the facts presented in part II of this series   In this it was pointed out that *X begoniae* (pathogenic on begonia), *X cucurbitae* (pathogenic on field

the variant strain, cultures were transferred in series into nutrient broth at 24-hour intervals   Twenty transfers were made   After each transfer the presence of creatinase was ascertained by inoculating a loopful of the nutrient broth culture into phosphate-buffered creatinine solution   The enzyme activity was not lost, but declined gradually as manifested in a delay of growth for 48 hours and in its limitation mainly to the surface of the medium

Three stock culture strains of *P   aeruginosa* and two stock culture strains of *P   fluorescens* failed to grow in phosphate-buffered creatinine solution   An attempt was made to adapt one of the strains of *P   aeruginosa* to this medium The organism was inoculated into nutrient broth containing 0 2 per cent of crea tinine and was transferred in series at 24-hour intervals for 12 successive days No measurable breakdown of creatinine took place, nor did growth occur in phosphate-buffered creatinine solution inoculated from the last one of the 12 serial transfers

In another experiment successive transfers in decreasing amounts of nutrient broth, brought up to volume with distilled water, were made   The creatinine concentration was kept constant at 0 2 per cent   A method of bacterial adapta tion described by Hegarty (1939) was used   Transfers were made in series every 90 minutes, at which time the end of the lag phase of *P   aeruginosa* was assumed to have been reached   All tubes were inoculated heavily   Six serial transfers were made   Tests for creatinase activity of the bacteria present in the last tube gave negative results

Finally a heavy inoculum of the stock culture strain was streaked on several plates of creatinine phosphate agar medium and incubated at room temperature for 2 weeks   No growth occurred   It would seem that the strain used was not adaptable to creatinine under the experimental conditions outlined

Enzyme specificity and growth requirements   Kopper and Beard (1947) observed that the creatinine-decomposing enzyme of the atypical strain acted on creatinine, creatine, and glycocyamidine, but failed to attack hydantoin   Phos phate-buffered solutions containing 0 1 per cent creatine, glycocyamidine, and hydantoin, respectively, were prepared and inoculated with the organism Growth developed in creatine but not in glycocyamidine or hydantoin   In or der to determine a possible cause for the discrepancy between the action of the enzyme on glycocyamidine and the failure of the strain to reproduce on this substrate, an attempt was made to grow the organism on the hydrolytic products of creatinine and glycocyamidine   These two chemical compounds are internal anhydrides of creatine (NH$_2$ C   N CH$_2$ COOH) and glycocyamine (NH$_2$ C
                                                                    || | ||
                                                                   NH   CH$_3$ NH

NH CH$_2$ COOH), respectively   The products of the hydrolysis of creatine would be urea and sarcosine, of glycocyamine urea and glycine   Phosphate-buffered solutions of 0 1 per cent urea, sarcosine, and glycine, respec tively, were prepared and inoculated with cultures of the atypical stu and of three strains of *P   aeruginosa* and two strains of *P   fluorescens*   N growth took place in urea   Sarcosine supported adequately the growth of

strains Glycine proved to be a poor medium The organisms either failed to multiply in it or did so only slightly after prolonged incubation This would seem to present additional evidence for the hydrolytic action of the creatinine-decomposing enzyme of the atypical strain The enzyme may effect the splitting of C   N and C   N linkages, but growth can only be supported by the

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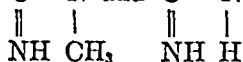
resulting split products On a good nutrient such as sarcosine the organisms multiply readily, which leads to the production of more enzyme, which in its turn causes a further breakdown of creatinine or creatine, whichever is the substrate, and a greater accumulation of sarcosine On a poor nutrient such as glycine, on the other hand, reproduction is so slow that no proper chain reaction can develop, which may account for the inadequacy of glycocyamidine as the sole source of carbon and nitrogen in a culture medium for the growth of the atypical strain

Preservation of cultures of the atypical strain Cultures of the atypical strain were kept on creatinine phosphate agar slants at room temperature When bacteria were transferred from such slants to phosphate-buffered creatinine solution or nutrient broth after 8 to 10 days, they failed to grow The organisms lost their viability also on nutrient agar, to which 2 per cent creatinine had been added, within the same length of time Their creatinase activity, however, was unaffected, as shown by the disappearance of creatinine from solutions incubated with suspensions of the dead organisms Both viability and creatinase activity could be preserved by keeping cultures on nutrient agar slants aerobically or on creatinine phosphate agar slants under oil This was proved by transfers from such slants after 45 and 60 days, respectively Work is now in progress to investigate the cause of the delayed lethal effect of creatinine agar media on cultures of the atypical strain under aerobic conditions

DISCUSSION

Karström's differentiation of bacterial enzymes into "constitutive" and "adaptive" enzymes was enlarged upon by Krebs and Eggleston (1939), who subdivided the latter into "partially adaptive" enzymes, which are formed in the absence of the specific substrate but increased in its presence, and "totally adaptive" enzymes, which are formed only in the presence of the specific substrate The creatinine-decomposing enzyme described by Dubos and Miller (1937) was shown to be "totally adaptive " Evidence presented in this study would favor the classification of the creatinase of the atypical strain of *P aeruginosa* as a "partially adaptive" enzyme One can only speculate on the mode of origin of such enzymes They may arise from mutations of the parent strain, which are of a more fundamental character than those producing "totally adaptive" enzymes This would explain the greater difficulties encountered in inducing them

As pointed out by Luria (1947), most bacterial classifications are only determinative keys, which cannot be compared with the well-defined systems of zoological and botanical taxonomy Many bacterial species and even genera

are separated on the basis of character differences that may be brought about by a single mutational step   The organism described here may have arisen in such a way, since aside from its creatinase activity it is indistinguishable from the species *P aeruginosa*

SUMMARY

A strain of *Pseudomonas aeruginosa*, first isolated from urine, was cultivated in a phosphate-buffered solution containing creatinine as the sole source of carbon and nitrogen

The strain possesses a specific creatinine-decomposing enzyme, creatinase, which is not lost after 20 successive transfers through nutrient broth without creatinine

Attempts to adapt a stock culture strain of *P aeruginosa* to grow in a phosphate-buffered creatinine solution were unsuccessful

The creatinase acts on glycocyamidine, but this compound is inadequate for serving as the sole source of carbon and nitrogen for the growth of the atypical strain

Sarcosine, a hydrolytic product of creatinine and creatine, represents a good culture medium for the atypical strain and five other strains of *P aeruginosa* and *Pseudomonas fluorescens* tested   Glycine, a hydrolytic product of glycocyamidine, is a poor nutrient for all strains

Cultures of the strain could be preserved on nutrient agar aerobically or on 2 per cent creatinine phosphate agar under oil for 45 and 60 days, respectively

Cultures kept aerobically on creatinine phosphate agar or on 2 per cent creatinine nutrient agar lost their viability but not their creatinase activity within 8 to 10 days

The nature of the enzyme and its possible mode of origin are discussed

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THE CORRELATION BETWEEN THE INHIBITION OF DRUG RESISTANCE AND SYNERGISM IN STREPTOMYCIN AND PENICILLIN[1]

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The problem of drug resistance has become an important limiting factor in the therapeutic efficiency of streptomycin (Buggs *et al*, 1946, Finland *et al*, 1946, Bondi *et al*, 1946) We have previously shown in the case of streptomycin that of 13 strains tested all had the ability to throw off, spontaneously, variants resistant to streptomycin (Klein and Kimmelman, 1946, Klein, 1947) The destruction by streptomycin of the mass of susceptible bacteria and the multiplication of the few highly resistant variants was indicated to be a mechanism for the development of streptomycin resistance Alexander and Leidy (1947), working with *Hemophilus influenzae*, have recently obtained similar results Clinically, the inhibition of the rapid development of streptomycin resistance may then require the destruction of a relatively small number of resistant bacteria, which might be effected by the addition of a low concentration of another drug In the present work we have therefore studied the combined action of streptomycin, penicillin, and sulfadiazine *in vitro* and determined the relationship between the synergistic action of the compounds and the inhibition of the development of streptomycin resistance

MATERIALS AND METHODS

Staphylococcus aureus, susceptible to streptomycin, penicillin, and sulfadiazine, was used as the test organism A casein hydrolyzate medium (Strauss, Dingle, and Finland, 1941) containing 0 5 per cent glucose provided a clear medium which was convenient in the determination of growth rates turbidimetrically in the Klett-Summerson photoelectric colorimeter The presence of the glucose resulted in a drop in pH after 24 hours that did reduce the streptomycin activity (Geiger, Green, and Waksman, 1946) However, this did not interfere with the interpretation of the results on the combined drug action

The tests for drug activity were performed as follows Six ml of the casein hydrolyzate medium, containing the various drugs singly or in combination, were added to the Klett-Summerson tubes, and a standard inoculum of 0 1 ml of a 20- to 24-hour culture, diluted to give a reading of 50 on the Klett-Summerson colorimeter (approximately 15,000,000 bacteria), was seeded into each of the tubes This large inoculum provided a rapid initial growth, which permitted the taking of turbidity readings at 6 hours, in addition to the 12-, 24-, and 48-hour readings In preliminary assays it was found that the 24-hour growth

curves obtained on the basis of turbidity readings followed essentially the same pattern as the 24-hour growth curves obtained on the basis of viable counts The 48-hour readings were included to show any delayed growth obtained with the inhibitors

The increase in drug resistance was estimated after 48 hours' incubation at 37 C   The bacteria were subcultured from the initial drug assays after 48 hours and grown for 20 to 24 hours in the casein hydrolyzate medium   A standard 0 1-ml sample was inoculated into the same drug concentration initially used, and growth was again determined turbidimetrically   The increase in the growth rate was then a measure of the increase in resistance to the drug   Turbidity readings of the medium, plus the standard inoculum, were taken at the beginning of all experiments, and the increase in turbidity over the initial reading was recorded   Only increases in turbidity greater than a reading of 10 were recorded in the graphs, and the turbidity readings were plotted as ordinates on a log scale All growth curves are representative experiments from at least four separate assays

<div align="center">RESULTS</div>

In figure 1 are shown the growth rates of the initially susceptible bacteria grown in partially inhibitory concentrations of penicillin, streptomycin, and sulfadiazine   The increase in resistance to each of the drugs is indicated for the 48-hour subcultures reassayed against the same concentration of the respective drugs   The bacteria subcultured after 48 hours from the initial assays of each of the drugs, and retested against the same concentration of each drug, showed a sharp increase in streptomycin resistance, a moderate increase in penicillin resistance, and no increase (frequently a slight decrease) in the rate of growth in the presence of sulfadiazine   The increase in resistance after 48 hours to one drug did not result in an increase in resistance to any of the other drugs

We have found that the rate of increase in resistance to penicillin and streptomycin is a function not only of the specific drug but of the concentration of the drug used   It was found that the greater the partially inhibitory action of penicillin or streptomycin, the greater the increase in resistance   When bacteria were grown in 2 units of streptomycin per ml, subcultured after 48 hours, and retested against 8 units of streptomycin, they showed only a relatively small increase in resistance   Bacteria grown in 4 and 8 units of streptomycin per ml showed significantly greater increases in resistance   Likewise, bacteria subcultured after 48 hours' growth in 0 02 of a unit of penicillin per ml and retested against 0 06 units of penicillin per ml showed only a slight increase in resistance when compared with the increase in penicillin resistance of bacteria grown in 0 04 and 0 06 units of penicillin per ml   This role of drug concentration in the development of penicillin and streptomycin resistance can be explained on the basis of the selection and multiplication of resistant variants   In high concentrations of penicillin or streptomycin which did not completely inhibit growth all but a few of the most resistant bacteria in the initial inoculum would be eliminated   These few bacteria could multiply and resistant variants would

thrown off in the direction of greater drug resistance   However, at lower drug concentrations one would not obtain so effective a selection of the few resistant variants, more of the less resistant bacteria would survive and on subculture they would tend to overgrow the few most resistant variants   Upon reassaying such a culture would show only a moderate or slight increase in resistance   We also observed that when there was no significant inhibition by
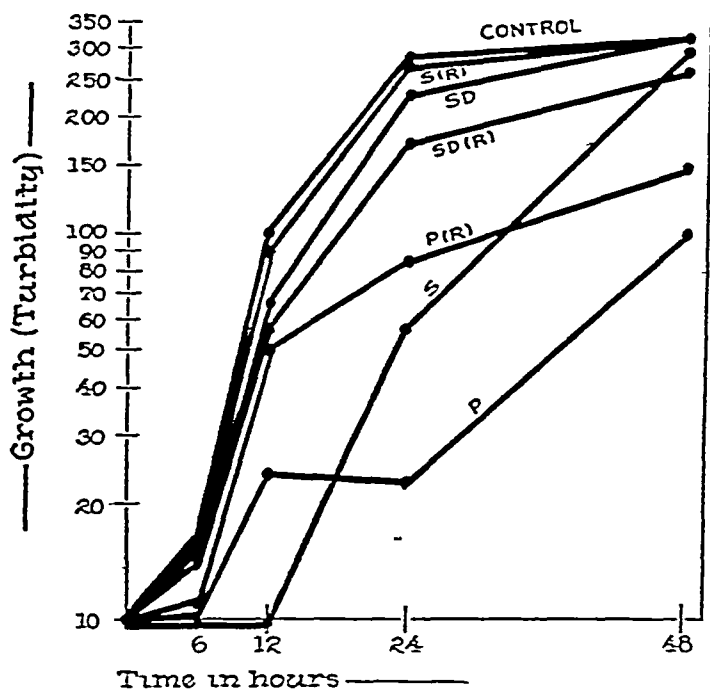

FIG 1 DEVELOPMENT OF RESISTANCE TO STREPTOMYCIN, PENICILLIN, AND SULFADIAZINE (STAPHYLOCOCCUS AUREUS)

Medium casein hydrolyzate, pH 7 4   Inoculum 0.1 ml of 20- to 24-hour culture (approx 15,000,000 organisms)   P = penicillin, 0 06 u/ml seeded with susceptible bacteria   S = streptomycin, 8 0 u/ml seeded with susceptible bacteria   SD = sulfadiazine, 1 5,000 seeded with susceptible bacteria   P(R) = penicillin, 0 06 u/ml seeded with organisms grown for 48 hours in 0 06 u/ml penicillin   S(R) = streptomycin, 8 0 u/ml seeded with organisms grown for 48 hours in 8 0 u/ml streptomycin. SD(R) = sulfadiazine, 1 5,000 seeded with organisms grown for 48 hours in 1 5,000 sulfadiazine

the drug i e , when no selection of the more resistant forms would occur there was no demonstrable increase in resistance

Combined action of two drugs and the inhibition of streptomycin resistance We determined the relative effectiveness of sulfadiazine and penicillin when added to streptomycin both with respect to their ability to increase the inhibitory action of streptomycin and their effectiveness in decreasing the streptomycin resistance of bacteria surviving the action of the drug   The results are shown in figures 2 and 3   When 1 5 000 sulfadiazine or 0 06 of a unit of penicillin were added to 8 units of streptomycin the combined action of the two drugs was

greater than either drug alone and the effect was not a simple additive one Sulfadiazine in a 1 5,000 concentration, which was less inhibitory than 0 06 units of penicillin, was far more effective when combined with streptomycin than was penicillin, and this greater effectiveness of sulfadiazine as a synergist was related to its ability to reduce more effectively the resistance of bacteria surviving the action of streptomycin   In figure 3 is shown the increase in resistance of bacteria surviving the action of (1) 8 units of streptomycin, (2) 8 units of streptomycin plus 0 06 units of penicillin, and (3) 8 units of streptomycin plus 1 5,000 sulfadiazine   The bacteria grown in streptomycin alone showed a
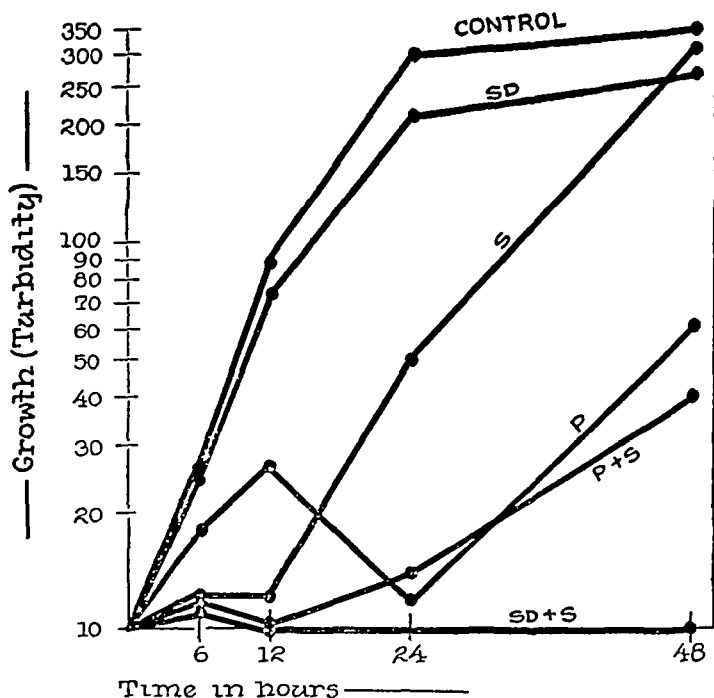

FIG 2 INHIBITORY ACTION OF STREPTOMYCIN, PENICILLIN, AND SULFADIAZINE—
SINGLY AND COMBINED—ON SUSCEPTIBLE STAPHYLOCOCCUS AUREUS
P = penicillin, 0 06 u/ml   S = streptomycin, 8 0 u/ml   SD = sulfadiazine, 1 5,000
P + S = penicillin, 0 06 u/ml plus streptomycin, 8 0 u/ml (final concentrations)   SD + S
= sulfadiazine, 1 5,000 plus streptomycin, 8 0 u/ml (final concentrations)

very marked increase in resistance, and the bacteria grown in the combination of streptomycin and penicillin showed almost the same increase in streptomycin resistance   However, the bacteria grown in streptomycin and sulfadiazine showed only a moderate increase in streptomycin resistance, indicating that the greater activity of sulfadiazine as a synergist was associated with a greater activity in inhibiting the development of streptomycin resistance   There was, however, in all cases an increase in streptomycin resistance over the initial susceptibility of the bacteria

When two drugs are combined, each inhibits the development of drug re-

sistance to the other   Streptomycin was found to inhibit effectively the development of penicillin resistance, as did sulfadiazine   No increase in sulfadiazine resistance was ever observed after 48 hours when subcultures were tested from sulfadiazine alone or in combination with other drugs

Combined action of three drugs   The combined action of 1 10,000 sulfadiazine, 0 04 units of penicillin, and 4 units of streptomycin was determined against *S aureus*, and the results are shown in figure 4   The combination of streptomycin and sulfadiazine or the combination of streptomycin and penicillin effected
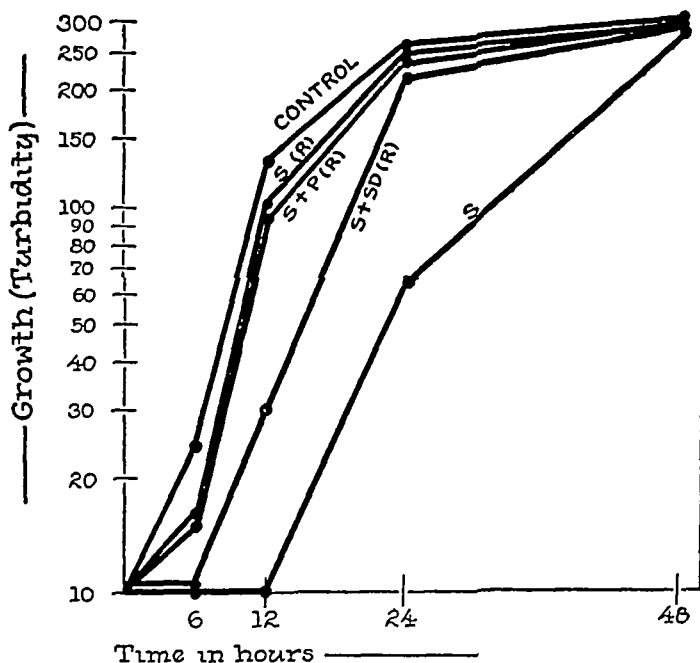

FIG 3 INCREASE IN STREPTOMYCIN RESISTANCE OF BACTERIA PREVIOUSLY GROWN IN STREPTOMYCIN, STREPTOMYCIN-PENICILLIN, STREPTOMYCIN-SULFADIAZINE (STAPHYLOCOCCUS AUREUS)

S = streptomycin, 8 0 u/ml seeded with susceptible bacteria   S(R) = streptomycin, 8 0 u/ml seeded with organisms grown for 48 hours in 8 0 u/ml streptomycin   S + P(R) = streptomycin, 8 0 u/ml seeded with organisms grown for 48 hours in 8 0 u/ml streptomycin plus 0 06 u/ml penicillin (final concentrations)   S+SD(R) = streptomycin, 8 0 u/ml seeded with organisms grown for 48 hours in 8 0 u/ml streptomycin plus 1 5,000 sulfadiazine (final concentrations)

only a partial inhibition of growth, whereas the combination of all three drugs completely inhibited growth   Though inhibition was complete, there were always a few bacteria surviving the combined drug action   These bacteria when subcultured and reassayed against streptomycin, penicillin, and sulfadiazine, respectively, never showed any increase in resistance and regularly showed a slight decrease in growth rate in the presence of streptomycin and occasionally a slight decrease in resistance to penicillin and sulfadiazine   The absence of any increase in resistance can be interpreted as being due to the

prompt inhibition of all multiplication by the three drugs with the subsequent inability of resistant variants to arise   The few surviving bacteria can be considered as nondividing cells in a physiological state temporarily unaffected by the action of the drugs

When the three drugs were combined in lower concentrations which permitted some multiplication, e g , penicillin 0 02 units per ml, streptomycin 4 units per ml, and 1 15,000 sulfadiazine, the bacteria when reassayed after 48 hours showed neither an increase nor a decrease in resistance   It should be pointed out again that lowering the test concentration of penicillin or streptomycin is in itself a
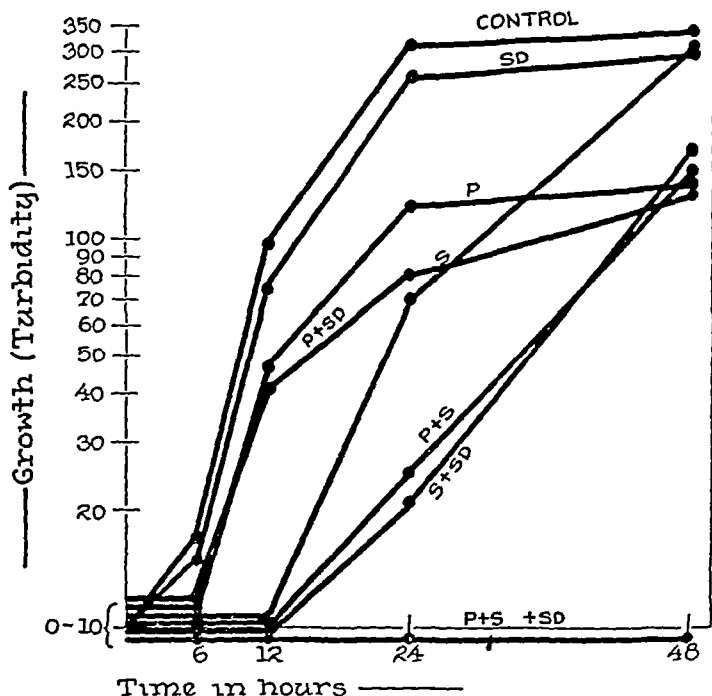

FIG 4 INHIBITORY ACTION OF PENICILLIN, STREPTOMYCIN, AND SULFADIAZINE
SINGLY AND IN COMBINATION ON SUSCEPTIBLE STAPHYLOCOCCUS AUREUS
P = penicillin, 0 04 u/ml   S = streptomycin, 4 0 u/ml   SD = sulfadiazine, 1 10,000
P+S = penicillin, 0 04 u/ml plus streptomycin, 4 0 u/ml (final concentrations)   P+SD
= penicillin, 0 04 u/ml plus sulfadiazine, 1 10,000 (final concentrations)   S+SD = strepto
mycin, 4 0 u/ml plus sulfadiazine, 1 10,000 (final concentrations)   P+S+SD = penicillin,
0 04 u/ml plus streptomycin, 4 0 u/ml plus sulfadiazine, 1 10,000 (final concentrations)

factor in effecting a decrease in the rate of development of drug resistance   However, this reduction in drug concentration is not in itself sufficient to eliminate completely the development of drug resistance in the case of the individual compounds

It should be pointed out that both drugs must be present in concentrations which are in themselves inhibitory   We have found that if one exposes a streptomycin-resistant culture to streptomycin and sulfadiazine, or streptomycin and penicillin, one obtains the inhibitory action of the sulfadiazine or penicillin alone

DISCUSSION

The relationship between synergism and drug resistance has been previously indicated by us in a report on the combined action of penicillin and the sulfonamides (Klein and Kalter, 1945)   It was found that an important factor in the observed synergism was the ability of a small amount of an added drug, in this case the sulfonamide, to prevent the multiplication of the few bacteria resistant to the test concentration of penicillin

Several factors may be considered in the present inhibition of drug resistance resulting from the simultaneous use of several drugs   We have already indicated that the use of lower concentrations of each drug is an important factor in the decreased resistance to the drug   Carpenter, Bahn, Ackerman, and Stokinger (1945) found that when bacteria were grown in sulfathiazole, rivanol lactate, promin, and penicillin, drug resistance did not develop against any of the compounds   In the combination of four drugs Carpenter and his coworkers used one-fourth the drug concentration initially used in the development of resistance to each agent   It would be of interest to know to what extent this reduction in the concentration of the individual drugs was related to the elimination of resistance when all four drugs were combined

As a synergist with streptomycin, the greater activity of sulfadiazine as compared with penicillin may be related to the very high degree of sensitivity of sulfadiazine to the total number of bacteria present   We have found, for example, that a 1,000-fold decrease in the size of our S $aureus$ inoculum increased the sulfadiazine titer over 30-fold, but under similar conditions the penicillin titer was increased only 3-fold   Hence when only a small number of streptomycin-resistant cells are present, low concentrations of sulfadiazine would be particularly effective

If one assumed that a drug had an all or none effect, i e , it either inhibited a bacterium from dividing or left the cell essentially unaltered, then the combined effect of the drugs could be explained exclusively in terms of this independent action   A given concentration of streptomycin would therefore destroy all but a small number of bacteria completely resistant to it and the small concentrations of the added drug or drugs would independently inhibit the small number of surviving bacteria   If, however, a drug can significantly modify cellular metabolism though not inhibit cell division, then it is possible that two drugs acting on a single cell may together effect complete inhibition or killing when each alone could not (Mudd, 1945)   One would then have in addition to the independent action, which must occur, this combined action on a single cell

Apart from any consideration as to the precise mode of action and with due regard to possible toxic effects, one can state that drugs having some limited degree of action against streptomycin-resistant bacteria are potential tools for reducing or eliminating the development of streptomycin resistance   It may be mentioned that antibodies and phagocytes should play a role in inhibiting the development of resistance by suppressing the multiplication of resistant cells   It is of particular interest to note that Schnitzer, Lafferty, and Buck (1946) found that drug resistance of the trypanosomes developed most rapidly in those treated experimental animals in which there was little antibody activity

SUMMARY

After 48 hour's growth in a casein hydrolyzate medium containing streptomycin, penicillin, or sulfadiazine, *Staphylococcus aureus* showed a marked increase in resistance to streptomycin and penicillin and no increase in resistance to sulfadiazine

The greater the partially inhibitory concentration of streptomycin or penicillin the greater the increase in the rate of development of resistance

Sulfadiazine, when added to streptomycin broth, was far more effective as a synergist and inhibitor of streptomycin resistance than was penicillin

Low concentrations of streptomycin, penicillin, and sulfadiazine when combined were highly effective in inhibiting multiplication and prevented the development of drug resistance

The results are interpreted on the basis of the selection and inhibition of resistant variants

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ORAL IMMUNITY TESTS OF DYSENTERY ANTIGEN IN WHITE MICE

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Lilly Research Laboratories, Indianapolis, Indiana

A current publication by Cooper and Keller (1947) on oral dysentery immunity in mice prompts us to submit some results on the same subject   Slightly different bacillary preparations were used

The present report gives results we have obtained by applying the Grasset technique to dysentery bacilli, including Shiga, Hiss, Flexner, Sonne, and Mt Desert types   Grasset (1939) prepared typhoid "endotoxoid" by repeatedly freezing and thawing typhoid bacillus suspensions, and subsequently treating the resultant lysate with formalin to reduce the toxicity   Human doses of three or four times the usual size may be given with little discomfort, and a superior degree of immunity is claimed   Using the same technique, we have found that formalin treatment of repeatedly frozen and thawed dysentery bacillus lysate for about a month decreased the toxicity considerably as judged by intraperitoneal tests in mice   Table 1 indicates a fourfold or more (possibly 32-fold) decrease in toxicity in 26 days of formalinization at 37 C

Following such detoxification with formalin, the culture lysates, either as single types or equal part mixed types, were usually adsorbed on starch and desiccated so that 1 gram represented 60,000 million original bacilli   The technique for oral immunity tests was that previously described (Powell, 1942) but modified by the use of white mice to assay the potency of this antigen Following oral immunization of white mice, decimal dilutions of 18-hour agar slant cultures suspended in a fluid containing 5 per cent gastric mucin and 0 2 per cent agar were given intraperitoneally for challenge to both treated and control mice   Since the virulence of the cultures for control mice was from 10^{-8} to 10^{-10} part of an agar slant, there was ample opportunity to detect active immunization of test mice with infecting doses of culture much less than primarily toxic doses

Preliminary experiments with different doses of single type and mixed type antigens, both in moist and dry condition, showed that (a) both homologous and slight heterologous type immunity can be produced orally in white mice, (b) antigen derived from 20,000 million bacilli appeared to be the best oral dose, being sufficiently strong to incite immunity and without harmful action on mice—10,000 million bacilli appeared insufficient and 50,000 million appeared somewhat toxic, (c) 10 oral doses, each derived from 20,000 million bacilli, given to mice in 5 days, i e , 2 doses per day, sufficed at 1 week after the last dose to incite immunity against 1, 10, and sometimes 100 MLD of culture suspended in the mucin-agar enhancement fluid, and (d) 20 doses of half-size were the equivalent of (c)   The degree of oral immunity attained here is quantitatively

about the same as that observed in mice by Felsen and Osofsky (1938) with injectable vaccine, and successful immunization against great multiples of a fatal dose of living dysentery bacilli has not been reported

A lot of antigen representing 12,000 million bacilli of each of the five types referred to above, or a total of 60,000 million bacilli per gram in starch, has been tested orally in 95 white mice against the five types of infections   On account of the bulkiness of the starch vehicle we used the sequence of doses indicated under (d) above   The results of these tests are recorded in table 2   The various

TABLE 1

Detoxification of dysentery bacillus antigen

INTRAPERITONEAL MOUSE DOSE 0 5 ML	FORMALINIZED ANTIGEN INCUBATED AT 37 C (DAYS)				
	0	5	12	19	26
Undiluted	1*	1	1	1	5
Diluted					
1 2	1	1	1	1	4
1 4	2	S	4	4	5
1 8	1	3	4	4	6
1 16	3	5	4	S	S
1 32	2	S	S	S	S

* Legend each figure indicates day of death of a mouse, S indicates survival at 7 days

TABLE 2

Oral immunity tests of dysentery antigen in white mice against five types of dysentery bacilli

FRACTION OF A SLANT OF DYSEN TERY CULTURE IN JECTED IN 0.5 ML	SHIGA		HISS		FLEXNER		MT DESERT		SONNE	
	Immun ized	Controls	Immun ized	Controls	Immun ized	Controls	Immun ized	Controls	Immun ized	Controls
10⁻⁵	DDDS	—	DDD	—	—	—	—	—	DDDS	—
10⁻⁶	DDDD	DDD	DDD	DDD	—	—	—	—	DDSS	DDD
			DDD							
10⁻⁷	DSSS	DDD	DDS	DDD	DD	DD	DS	DD	DDSSS	DDD
			DDS							
10⁻⁸	SSSS	DDD	DDD	DDD	DD	DD	DD	DD	DDSSS	DDS
			DDS		DS		DS			
10⁻⁹	—	SSS	DDD	DDS	DDDD	DDD	DDDD	DDD	—	S S
			SSS		SS	DD	DSSD	DDDD		
10⁻¹⁰	—	—	—	—	—	—	DDD	DDD	—	—
							SS	DD		

Legend D ═ mouse dead within 3 days S ═ mouse surviving — ═ test not done

test cultures, in decimal dilution in the mucin-agar virulence enhancement fluid, exhibited a high degree of virulence for normal control mice   Comparison in immunity between groups of treated and control mice injected with each of the series of live culture dilutions may be made in the horizontal columns in the table   Considerable immunity is exhibited toward the Shiga and Sonne types, and weaker immunity is exhibited against the Hiss, Flexner, and Mt Desert types A higher bacterial "count" in the mixed type vaccine is difficult to use owing to the over-all residual toxicity

It is concluded that dysentery antigen, after repeated freezing and thawing, may be detoxified considerably with formalin, and oral assay of this antigen can be done in a period of 5 days of immunization, 7 days of waiting, then 2 or 3 days for completing live-culture tests, or about 2 weeks in all   The oral response in mice appears to be better against the Shiga and Sonne types than against the Hiss, Flexner, and Mt Desert types   Possibly variable doses of the different types of organisms in the vaccine might have improved the results

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about the same as that observed in mice by Felsen and Osofsky (1938) with injectable vaccine, and successful immunization against great multiples of a fatal dose of living dysentery bacilli has not been reported

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1 4	2	S	4	4	5
1 8	1	3	4	4	6
1 16	3	5	4	S	S
1 32	2	S	S	S	S

* Legend   each figure indicates day of death of a mouse, S indicates survival at 7 d₃

TABLE 2

Oral immunity tests of dysentery antigen in white mice against five types of dysentery bacilli

FRACTION OF A SLANT OF DYSENTERY CULTURE INJECTED IN 0 5 ML	SHIGA		HISS		FLEXNER		MT DESERT		SONNE	
	Immunized	Controls	Immunized	Controls	Immunized	Controls	Immunized	Controls	Immunized	Con
10⁻⁶	DDDS	—	DDD	—	—	—	—	—	DDDS	
10⁻⁶	DDDD	DDD	DDD DDD	DDD	—	—	—	—	DDSS	D
10⁻⁷	DSSS	DDD	DDS DDS	DDD	DD	DD	DS	DD	DDSSS	D
10⁻⁸	SSSS	DDD	DDD DDS	DDD	DD DS	DD	DD DS	DD	DDSSS	D
10⁻⁹	—	SSS	DDD SSS	DDS	DDDD SS	DDD	DDDD DSSD	DDD DDDD	—	C
10⁻¹⁰	—	—	—	—	—	—	DDD SS	DDD DD	—	

Legend   D = mouse dead within 3 days   S = mouse surviving   — = test not done

test cultures, in decimal dilution in the mucin-agar virulence enhancement exhibited a high degree of virulence for normal control mice   Comparisons immunity between groups of treated and control mice injected with each of series of live culture dilutions may be made in the horizontal columns in the Considerable immunity is exhibited toward the Shiga and Sonne types weaker immunity is exhibited against the Hiss, Flexner, and Mt Desert A higher bacterial "count" in the mixed type vaccine is difficult to use to the over-all residual toxicity

PRODUCTION OF ASPERGILLIC ACID BY SURFACE CULTURES OF ASPERGILLUS FLAVUS

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It has been reported by White (1940), Rake, McKee, and Jones (1942), White and Hill (1943), Jones, Rake, and Hamre (1943), and Bush, Dickison, Ward, and Avery (1945) that the fungus *Aspergillus flavus* produces an antibiotic substance known as aspergillic acid *Aspergillus flavus* is also known to produce other antibiotic substances such as flavacidin (McKee, Rake, and Houck, 1943, McKee and MacPhillamy, 1943), and flavicin (Bush, Goth, and Dickison, 1945) However, for economical large-scale production of aspergillic acid, it was necessary to try additional media and methods of cultivation The studies described in this report resulted in the development of suitable methods for obtaining valuable increases in the yields of material produced by the fungus

METHODS OF CULTIVATION

White and Hill reported yields of 0 005 to 0 07 mg of crude crystalline material per ml of medium when *Aspergillus flavus* grew in surface culture at 23 C on a solution containing 2 per cent tryptone and 0 5 per cent sodium chloride Rake *et al* reported yields of 0 1 to 0 25 mg of crystalline aspergillic acid per ml of medium of the same composition Bush *et al* reported yields of 0 3 mg of crude crystalline material per ml of a solution containing 2 per cent Difco peptone and 2 per cent lactose Similar results were obtained when the same media and methods were tried in this laboratory

In the attempt to increase the yields in this laboratory several modified medium formulas were tried Some were promising but others gave completely or nearly completely negative results Individual media containing suitable sources of necessary nutrients such as soybean meal, vegetable meal, casamino acid, veal broth, Czapek-Dox, neopeptone, corn steep liquor, Brewer's yeast, and proteose peptone produced no detectable amount of aspergillic acid A few other media which contained boiled potatoes, *dl*-isoleucine, Sabouraud's solution, or brain-heart infusion as the essential ingredient produced substantial amounts of aspergillic acid, but the one which gave the best yield as determined by assay was a simple solution containing 2 per cent Difco yeast extract and 1 per cent glycerol This medium on the average yielded 0 8 mg of aspergillic acid per ml of solution in actual large-scale production lots and assayed over 1 mg per ml in the case of some smaller, experimental lots

EXPERIMENTAL RESULTS

Fifty ml of the yeast extract glycerol medium were sterilized per 250-ml Erlenmeyer flask at 15 pounds for 15 minutes The initial pH range was 6 3 to

6 6   The inoculum was 10^{10} spores in 1 ml of spore suspension   Incubation was at 25 C   These experiments represent several dozen flasks—each individual flask having been assayed biologically and spectrophotometrically [1]

After inoculation of a flask, growth commenced promptly and by 48 hours a heavy, white, wrinkled pellicle was formed   The liquid under the pellicle was tested for activity and pH daily from the end of the third day until the twelfth day   The results of these experiments are shown in figures 1, 2, and 3
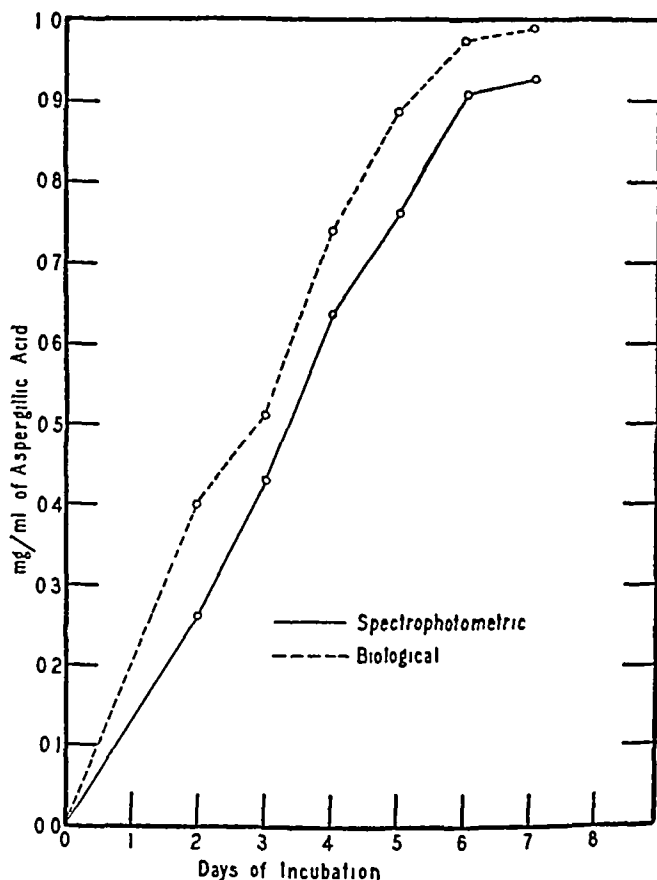
Fig 1 Aspergillic Acid—Assay vs Days

It will be noted from the graphs that as the pH rises the activity also increases, and on the average the optimum incubation period is 6 to 7 days   The rise in pH is fairly rapid and consistent   The peak activity, as a rule, is obtained when the pH reaches 7 8   The greatest rise in pH occurs during the first two days the pellicle is forming, and thereafter rises more slowly

similar results were obtained in 110 production lots of 200 one gallon bottles, each 7   approximately 300 ml medium   As each production lot was harvested after me   bation, it was pooled and assayed as such both biologically and spectrophoto-

METHOD OF ASSAY

Previously, Rake *et al* (1942, 1943) reported a rapid test for the activity of certain antibiotic substances, including aspergillic acid, based on the interference with the luminescence produced by luminescent bacteria   This interference ¹ can be directly correlated with antibacterial activity   However, in this laboratory two other methods were preferred   the spectrophotometric method which
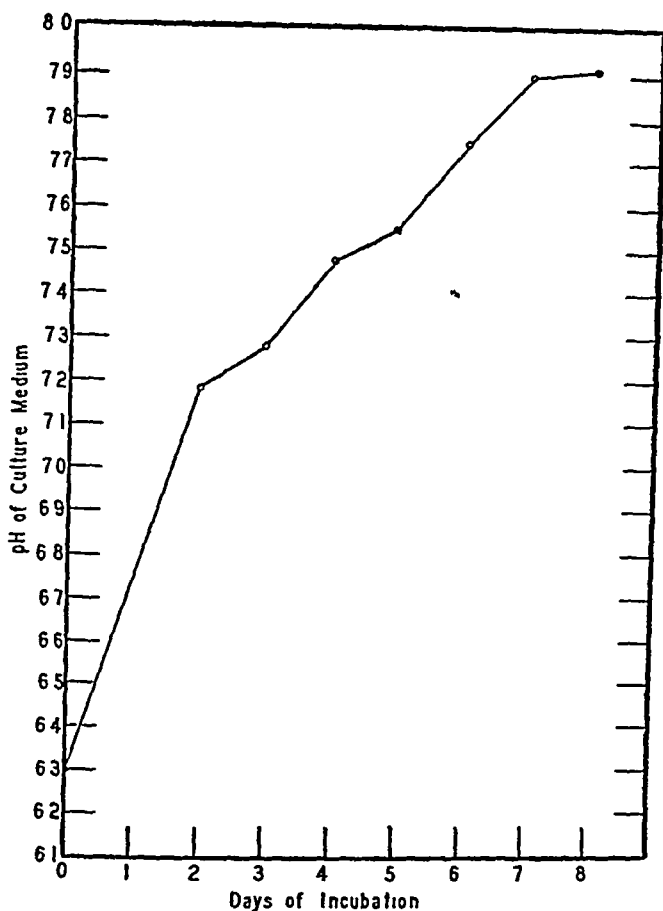

FIG 2   ASPERGILLIC ACID, pH CURVE

is based on the ultraviolet absorption curve for aspergillic acid (maximum at 336 mμ in buffer), and the biological method in which activity is tested against a standard solution of aspergillic acid (serial tube dilution)   The organism used in the latter test is the Heatley strain of *Staphylococcus aureus*

The two assays confirm each other (Dr J D Dutcher, to be published) Spectrophotometrically, it is impossible to differentiate between aspergillic acid and hydroxy-aspergillic acid   Biologically the two acids are completely dif-

ferent, the hydroxy-aspergillic acid being inactive, whereas the aspergillic acid is quite active   Physically and chemically the precipitated materials are not alike   Hydroxy-aspergillic acid has a melting point of 149 to 150 C and has 3 atoms of oxygen in its chemical structure, whereas aspergillic acid melts at 90 to 100 C and has but 2 atoms of oxygen in its chemical structure
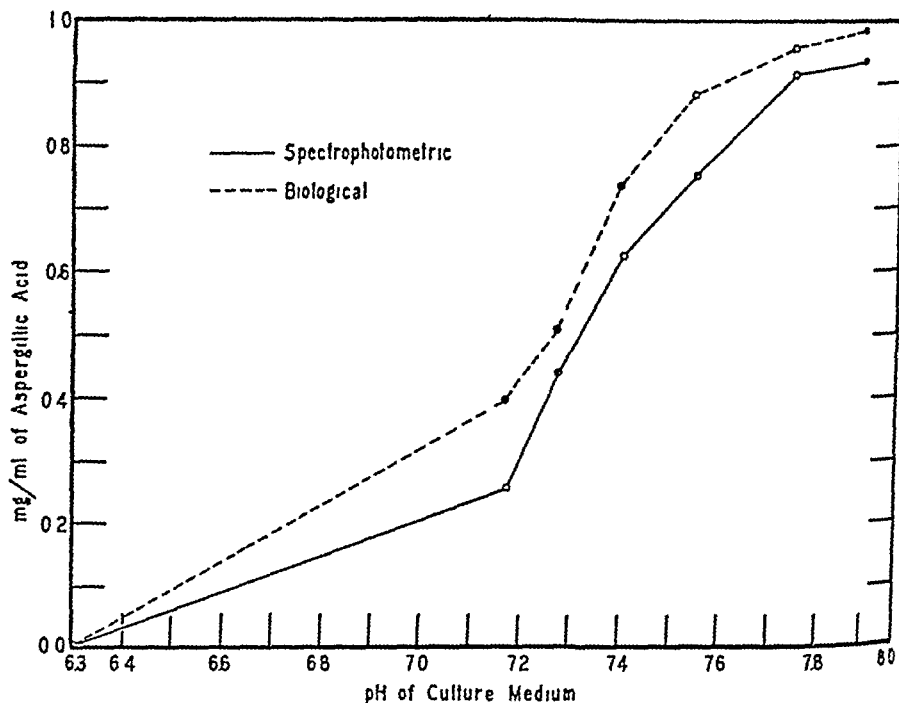

FIG 3 ASPERGILLIC ACID—ASSAY vs pH

TABLE 1

The effect of yeast on the yield of aspergillic acid

YEAST NO	INITIAL pH	FINAL pH	SPECTRO-ASSAY	BIO-ASSAY
			mg/ml	mg/ml
333607	6 55	8 8	1 01	0 76
380588	6 5	8 9	0 03	0 68
378856	6 4	8 5	0 07	0 81
382370	6 5	8 75	0 85	0 60
382097	6 5	8 5	0 92	0 80

If a high titer in crude broth is obtained biologically, this must be verified spectrophotometrically, since *Aspergillus flavus* also produces penicillinlike substances   If a high titer in crude broth is obtained spectrophotometrically, this must be verified biologically to make certain that hydroxy-aspergillic acid is not being produced   If a high titer is obtained in both the chemical and bio-assay, a relatively large amount of aspergillic acid may be expected in extraction or isolation process

It is a relatively simple matter to produce hydroxy-aspergillic acid, but aspergillic acid is more difficult to produce Experience has shown that not all lots of yeast extract will produce a high quantity of aspergillic acid

Several lots of Difco yeast extract were tested and from the results in table 1 it can be seen that production of aspergillic acid depends on the yeast that is in the medium

Yeasts 333607, 382370, and 382097 yielded the greatest amount of aspergillic acid, while yielding a small percentage of hydroxy-aspergillic acid Yeasts 380588 and 378856 yielded practically no aspergillic acid or hydroxy-aspergillic acid, but did cause the formation of a penicillinlike substance

SUMMARY

A method is described which enhances the production of aspergillic acid by *Aspergillus flavus* in surface cultures A simple solution containing 2 per cent Difco yeast extract and 1 per cent glycerol yielded the highest titers of approximately 0 8 mg per ml in the crude broth Emphasis is also placed on the importance of assay for aspergillic acid by both the spectrophotometric and the biological methods to verify the production of aspergillic acid, hydroxy-aspergillic acid, or penicillinlike substances

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ferent, the hydroxy-aspergillic acid being inactive, whereas the aspergillic acid is quite active   Physically and chemically the precipitated materials are not alike   Hydroxy-aspergillic acid has a melting point of 149 to 150 C and has 3 atoms of oxygen in its chemical structure, whereas aspergillic acid melts at 90 to 100 C and has but 2 atoms of oxygen in its chemical structure
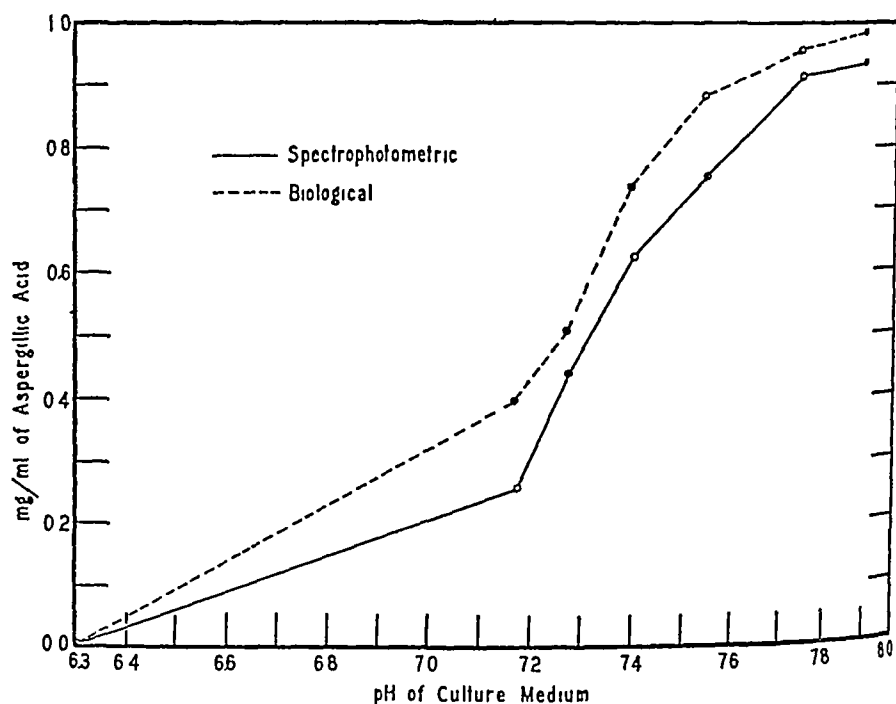

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CLOSTRIDIUM LACTO-ACETOPHILUM NOV SPEC AND THE ROLE OF ACETIC ACID IN THE BUTYRIC ACID FERMENTATION OF LACTATE

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Although the fermentation of lactate by butyric acid bacteria has been studied by a number of eminent bacteriologists (Schattenfroh and Grassberger, 1900, Bredemann, 1909, Boekhout and van Beynum, 1929), including Pasteur (1862) and Beijerinck (1893), it is still a very poorly understood process   A natural butyric acid fermentation of lactate has been reported to occur commonly in low grade silage (van Beynum and Pette, 1936), and, under laboratory conditions, several investigators have easily obtained crude enrichment cultures of butyric acid bacteria using lactate as the main substrate, but nearly all attempts to isolate and propagate these bacteria in pure culture on a lactate medium have failed   In pure culture the bacteria appear to lose their ability to attack lactate, although they can be cultured easily on sugar-containing media   Van Beynum and Pette (1935) finally succeeded in growing these organisms in a lactate medium, but only when an unusually high concentration of yeast autolysate was also provided   Consequently one cannot decide whether the lactate or the yeast autolysate provided the main carbon and energy source   Van Beynum and Pette rightly observe "There is much uncertainty about the lactate fermentation   As a matter of fact one does not know if it exists as a separate phenomenon, and very little is yet known about the relation between lactate and sugar fermentations "

In the present investigation we have used lactate-decomposing bacteria obtained by the enrichment culture method   By studying their nutritional requirements and chemical activities we have found why butyric acid bacteria cannot be grown in a simple lactate medium and have shown that acetic acid plays an important role in their metabolism

EXPERIMENTAL RESULTS

Enrichment and isolation of lactate-fermenting bacteria   The investigation was begun by the enrichment and isolation of lactate-fermenting butyric acid bacteria from soil   A medium of the following composition (medium 1) in grams per 100 ml was used   sodium lactate, 1, yeast autolysate, 0 3, $(NH_4)_2SO_4$, 0 05, $MgSO_4$ $7H_2O$, 0 01, K_2HPO_4, 0 05, $FeSO_4$ $7H_2O$, 0 002, pH 7, made up with tap water   The medium was inoculated with a small quantity of garden soil and incubated anaerobically at 37 C   Within 36 hours the medium became tur-

bid, considerable gas was given off, and the pH rose to 8 2 to 8 4   After 72 hours a determination of lactic acid by the method of Friedemann and Graeser (1933) showed that the lactate was completely decomposed

Several soils of different types were used as inocula for enrichment cultures of this type, and in every instance a vigorous fermentation was obtained within 30 to 48 hours   The predominant bacteria were always medium-sized, actively motile rods, a few of which usually contained oval, central, or subterminal spores In addition, a few small nonsporulating rods and vibrios were always present when the inoculum was not pasteurized   When a pasteurized soil inoculum was used, a more homogeneous but always less vigorous culture was obtained

In order to eliminate extraneous bacteria as far as possible before attempting the isolation of pure cultures, one or two successive transfers were made in the same enrichment medium   These cultures always developed within 30 to 40 hours, but were notably less vigorous than the original

Pure cultures were obtained without special difficulty from the enrichment cultures by the shake culture method of Burri (1902)   A solid medium of the same composition as the enrichment medium proved to be satisfactory   Oxygen was removed from the culture tubes by the use of a mixture of pyrogallol and potassium carbonate   Within 20 to 30 hours' incubation at 37 C, the agar in the more heavily inoculated tubes was split by gas, but at the higher dilutions a few well isolated colonies developed without apparent gas formation   After about 24 hours' incubation at 37 C colonies were visible up to the seventh or eighth dilution, but only at lower dilutions was the agar split by gas

The colonies of the lactate-decomposing bacteria are generally compact, fluffy, dark gray spheres composed of filamentous outgrowths   They are coarsely lobed and rough-edged, they eventually reach a diameter of 1 to 2 mm   The consistency of the colonies is such that the organisms can be easily drawn into a micropipette or transferred with an inoculating needle

By the repeated use of the shake culture method, nine strains were isolated in pure culture   Each was derived from a different soil

Morphological characteristics   All nine strains are very similar in appearance (figure 1)   The average cell dimensions are 0 8 by 4 6 microns, the variation in width being from 0 7 to 0 9 microns and in length from 3 to 8 microns   Spores develop after about 40 hours' incubation in a favorable medium, they are oval in shape and are usually located subterminally where they cause a distinct bulge in the cell   The average spore size is 1 1 by 1 5 microns   Young vegetative cells are actively motile by means of 20 or more peritrichous flagella   At this stage they are gram-positive   In old cultures most of the cells become gram negative   Cells generally occur singly or in pairs, though short chains can be seen   When first isolated, three strains appeared to possess small capsules, but on subsequent cultivation in a variety of media, no capsules could be observed in any of the strains

Physiological and cultural characteristics   All nine strains are similar in physiological characteristics   They are obligate anaerobes and their development is favored by the addition of a reducing agent such as sodium thioglycolate to the

medium The following compounds are readily fermented when supplied in a basal medium containing 3 volumes per cent yeast autolysate and the usual salts glucose, fructose, galactose, mannose, xylose, arabinose, rhamnose, lactose, sucrose, maltose, trehalose, raffinose, dextrin, glycogen, starch, xylan, mannitol, inositol, inulin, sorbitol, and dulcitol Glycerol and lactate are attacked very feebly in this medium However, if 0 8 per cent sodium acetate is also added, both glycerol and lactate are decomposed vigorously Acid and a moderate amount of gas are formed from the carbohydrates and polyalcohols The acid is generally a mixture of acetic and butyric acids (see below), and the gas is a mixture of hydrogen and carbon dioxide Iron milk is slightly acidified without

FIG 1 CLOSTRIDIUM LACTO ACETOPHILUM STRAIN 3, FORTY EIGHT HOUR CULTURE GROWN IN MEDIUM 1 WITH 0 1 PER CENT AGAR
Free spores are visible in the background ✕ 1,000

clotting, and a very small amount of gas is formed Nitrate is not reduced, indole is not formed in a glucose yeast autolysate medium A little hydrogen sulfide is formed

Strain 3, which was used for most of the later experimental work, grows at temperatures from 16 to 46 C The optimum appears to be close to 39 C The pH range is from 5 6 to 8 4, good growth occurring between pH 6 2 and 7 4 The organisms grow well in a mixed sodium and potassium phosphate buffer in concentrations up to 2 per cent, with 2 4 per cent buffer growth is perceptibly inhibited

Classification All the strains studied clearly belong to one species which is evidently closely related to *Clostridium butyricum* as defined by Bergey *et al*

(1939)   However, it is definitely stated that the latter species is unable to fer ment lactate   Two lactate-fermenting clostridia have been described in the liter ature, *Clostridium tyrobutyricum* of van Beynum and Pette (1935), and *Granu lobacter lactobutyricus* of Beijerinck (1893), but neither of these organisms appears to be able to attack the wide variety of carbohydrates and polyalcohols fermented by our strains   Beijerinck states that *G lactobutyricus* is unable to attack carbo hydrates at all   *C tyrobutyricum* is described as usually fermenting only glucose, fructose, and lactate   In view of the impossibility of identifying our organism with any previously described species, we have decided to call it *Clostridium lacto-acetophilum*, nov spec, for reasons which will appear below   Further work may prove that this organism is identical with some other species not now recog nized to be able to ferment lactate because the fermentation test was carried out in the absence of acetate (see below)   However, in the meantime the name *C lacto-acetophilum* will serve to identify lactate-fermenting butyric acid bacteria of the type we have described

Nutritional requirements   Shortly after pure cultures of *C lacto acetophilum* were first isolated, using a solid lactate yeast autolysate medium (medium 1), the organism was inoculated into a liquid medium of the same composition except for the absence of agar   Growth in this liquid medium was extremely sparse The addition of 0 1 per cent agar resulted in a slight improvement, and the addi tion of 2 per cent agar allowed moderately good growth   The failure of the organism to grow satisfactorily in liquid medium 1 could not be due to oxygen inhibition since the addition of 0 05 per cent sodium thioglycolate as a reducing agent did not cause any improvement

An attempt was made to improve the liquid lactate yeast autolysate medium by adding various substances to it, including larger amounts of yeast autolysate It soon became evident that growth in the liquid medium is almost proportional to the yeast autolysate concentration up to a level of about 30 volumes per cent This in itself would not be surprising were it not for the fact that 20 volumes per cent yeast autolysate are required to give as good growth in the liquid medium as can be obtained with only 3 volumes per cent in an otherwise identical solid medium

Previous experiments conducted in this laboratory (Barker, 1947, Bornstein and Barker) with another bacterium, *Clostridium kluyveri*, had shown that an abnormally high requirement for yeast autolysate may be due to a need for acetic acid, which is always present in yeast autolysate in small amounts   We therefore tried adding 0 3 per cent sodium acetate to the liquid lactate medium containing 3 volumes per cent yeast autolysate   The results were very striking, excellent growth occurred in the presence of acetate, whereas in its absence growth was extremely poor   Quantitative experiments on the relation between acetate con centration and growth measured with an Evelyn colorimeter showed that growth rate and the maximum cell yield increase with sodium acetate concentra tion up to about 0 8 per cent   Table 1 shows that the total amount of lac decomposed also increases with the initial acetate concentration

Several other substances were tested to determine whether they can subs'

for acetate in stimulating growth and lactate decomposition   The substances so tested were formate, propionate, butyrate, fumarate, succinate, malate, tartrate, citrate, pyruvate, ethanol, and glucose   They were used at concentrations of 0 1 and 0 5 per cent   Both lactate and lactate-acetate media were used as controls   It was found that only pyruvate can substitute for acetate in favoring both growth and lactate decomposition   Glucose, either alone or in combination with lactate, supports very good growth, but it does not accelerate the disappearance of lactate

It should be noted that *C   lacto-acetophilum* differs markedly from *C   kluyveri* with respect to the substrates that can be substituted for acetate   The latter organism can use propionate, butyrate, and to a lesser extent valerate, but cannot use pyruvate

TABLE 1

The dependence of growth and lactate decomposition on the acetate supply

INITIAL ACETATE	LACTATE DECOMPOSED	RELATIVE GROWTH
mM/100 ml	*mM/100 ml*	
0 05	0 44	177
0 79	1 89	446
1 52	2 89	680
2 26	3 78	809
3 00	4 67	888
3 73	5 22	982
4 47	5 78	1,107
5 94	6 22	1,177
7 40	6 55	1,192

Medium sodium lactate, 8 8 mM per 100 ml, yeast autolysate, 3 volumes per cent, the salts of medium 1, and the indicated sodium acetate concentrations   All cultures were incubated 3 days at 37 C under anaerobic conditions   Strain 3

When 0 6 to 0 8 per cent acetate is added to the culture medium, the concentration of yeast autolysate needed to give maximal growth is greatly reduced   About 3 volumes per cent is quite adequate, whereas at lower concentrations the cell yield decreases   However, the yeast autolysate level can be still further reduced to about 0 1 volume per cent without limiting growth if the medium is supplemented with 0 01 μg biotin and 10 μg *para*-aminobenzoic acid per 100 ml   A few attempts to replace yeast autolysate completely by known growth factors and amino acid mixtures were unsuccessful   In a medium containing lactate, acetate, biotin, *para*-aminobenzoic acid, and 0 01 volume per cent yeast autolysate, the organism failed to respond favorably to any of the following compounds or preparations thiamine, nicotinic acid, riboflavin, pyridoxine, folic acid, acid-hydrolyzed casein, peptone, and tryptone

The following medium (medium 2), which supports excellent growth of *C lacto-acetophilum*, was developed on the basis of the foregoing experiments   sodium lactate, 1 g, sodium acetate, 0 8 g, yeast autolysate, 0 5 ml (0 05 g dry

weight), sodium thioglycolate, 0 05 g, $(NH_4)_2SO_4$, 0 05 g, $MgSO_4$ $7H_2O$, 0 01 g, K_2HPO_4, 0 05 g, $FeSO_4$ $7H_2O$, 0 002 g, biotin, 0 01 μg, *para*-aminobenzoic acid, 10 μg, distilled water, 100 ml, pH 7   Heavier growth is obtained if lactate is replaced by glucose

Fermentation products   The observation that the amount of lactate decom posed is dependent upon the initial acetate concentration suggests that acetate is consumed in the lactate-acetate fermentation   This possibility was verified experimentally, and the products of the lactate-acetate fermentation in a growing culture were determined quantitatively by methods previously described (Barker and Haas, 1944)   The main products are butyric acid, carbon dioxide, and hydrogen (table 2, column 3)

The influence of acetate concentration on acetate utilization and butyrate for mation is shown in table 3   Only at the lowest initial concentration is there a

TABLE 2

Fermentation products

COMPOUND	ENRICHMENT CULTURE	PURE CULTURE STRAIN 3		
	Substrates			
	Lactate, 1%	Lactate 1% + acetate, 0.8%	Pyruvate 1%	Glucose, 1%
Acetic acid	50	−32	33	28
Butyric acid	35	65	33	73
Carbon dioxide	55 5	100	93	190
Hydrogen	10	59	30	182
Carbon recovery (%)	98	99	97	90
Redox index	1 39	1 05	0 96	1 12

The figures represent yields in moles per 100 moles of fermented substrate   Each me dium contained the salts of medium 1, 3 volume per cent yeast autolysate, and the indicated substrate concentrations   Cultures were incubated at 37 C until fermentation ceased

net production of both acetate and butyrate   At higher acetate concentrations there is always a disappearance of acetate, which increases with concentration It should be noted that added acetate is never entirely used up   The final con centration is always above 1 millimole per 100 ml   In this respect acetate be haves quite differently from most other substrates, like glucose and lactate, which under favorable conditions are completely fermented by this organism   The ex planation for this anomalous behavior appears to be that the utilization of acetate depends upon the concentration of butyrate   Column 5, table 3, shows that the butyrate-acetate concentration ratio in the fermented medium never exceeds a value of about 2 6 and is remarkably constant and independent of the   ⸱ acetate concentration over a wide range   This indicates the existence of ⸱ sort of equilibrium between acetate and butyrate   The ratio of butyrate f⸱ to acetate used (column 6, table 3) is more variable than the final concentr⸱

ratio   The former ratio is infinite at the lowest acetate level, indicating that all the butyrate is derived from lactate   At intermediate acetate levels, approximately one mole of acetate is used for each mole of butyrate produced   At higher levels, the molar quantity of acetate used considerably exceeds the butyrate formed   Since it is theoretically impossible to use more than one mole of acetate plus one mole of lactate in the formation of one mole of butyrate, this result must mean that some other product, such as acetone, is being formed under these conditions

The lactate-acetate fermentation is evidently analogous to the ethanol-acetate fermentation of C kluyveri   In the latter process, the ethanol is oxidized to an active form of acetic acid, which condenses with the substrate acetate to give an intermediate compound that is ultimately reduced to butyric acid (Barker,
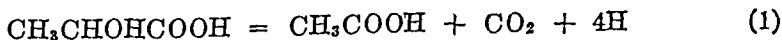
TABLE 3

Influence of acetate concentration on the lactate acetate fermentation

mm/100 ml					
Acetate			*Butyrate formed*	FINAL BUTYRATE / FINAL ACETATE	BUTYRATE FORMED / ACETATE USED
Added	Final	Used			
0 05	0 41	−0 36	0 295	0 72	∞
1 27	1 04	0 23	1 24	1 19	5 39
2 49	1 01	1 48	2 23	2 21	1 51
3 76	1 13	2 58	2 95	2 62	1 02
4 93	1 50	3 43	3 90	2 60	1 14
6 15	1 82	4 32	4 74	2 60	1 10
7 37	2 03	5 34	5 27	2 60	0 99
8 59	2 14	6 45	5 55	2 60	0 86
9 81	2 25	7 56	5 85	2 60	0 77
11 03	3 93	7 10	4 72	1 61	0 66
12 25	5 20	7 05	3 47	0 67	0 49

Medium no 1 containing 3 volumes per cent yeast autolysate, 1 per cent sodium lactate, and the specified amount of sodium acetate was used The cultures were incubated for 5 days at 37 C until fermentation had ceased   Strain 3

Kamen, and Bornstein, 1945)   In the lactate-acetate fermentation of C lacto-acetophilum lactate is evidently oxidized to carbon dioxide and acetic acid or a derivative thereof   It will be noted that one mole of carbon dioxide is formed per mole of lactate fermented as is required of such a mechanism   As in the C kluyveri fermentation, butyric acid is probably formed by a condensation of two moles of acetic acid or related compound, followed by a reduction   The postulated fermentation mechanism may be schematically represented as follows

$$CH_3CHOHCOOH = CH_3COOH + CO_2 + 4H \qquad (1)$$
$$2H = H_2 \qquad (2)$$
$$2CH_3COOH + 4H = CH_3CH_2CH_2COOH + 2H_2O \qquad (3)$$
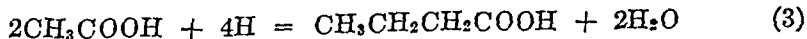
The need for an outside supply of acetate in the decomposition of lactate may be explained by considering the oxidation-reduction relations involved in the fer-

like *C butylicum*, *C acetobutylicum*, and *Butyribacterium rettgeri*, also use acetate as a primary oxidant, even though they do not need to have it supplied from an outside source   In such organisms, accessory oxidants, such as carbon dioxide, butyric acid, or acetone, are available to supplement acetic acid   In addition gaseous hydrogen may be formed

Our results explain why previous investigators have had so little success in growing butyric acid bacteria on lactate media in pure culture   Their bacteria did not lose the ability to ferment lactate, as has commonly been supposed, they simply were unable to oxidize lactate in the absence of acetate   When acetate was unintentionally added, as was done by van Beynum and Pette (1935) through the use of large amounts of yeast extract, the bacteria grew and decomposed lactate   It seems likely that the ability to ferment lactate and glycerol is much more widely distributed among butyric acid bacteria than has been previously reported   At least some species that are now thought to be unable to attack lactate and glycerol will probably be found to do so when acetate is added to the test media   The addition of 0 5 per cent sodium acetate to all media for butyric acid bacteria will probably be found advantageous

SUMMARY

The isolation and characteristics of a lactate-fermenting butyric acid bacterium, *Clostridium lacto-acetophilum*, nov spec , are described   It is shown that the decomposition of lactate by this organism is dependent on the presence and simultaneous utilization of acetate   The role of acetate in butyric acid fermentations is discussed

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ANTIBIOTIC PRODUCTION BY MARINE MICROORGANISMS[1]

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The multiplication of most nonmarine bacteria is inhibited by sea water (Korinek, 1927) ZoBell (1941) found that only from 4 to 15 per cent as many bacteria from soil, sewage, and other fresh-water or terrestrial sources formed colonies on nutrient agar prepared with sea water as on a similar medium prepared with distilled water Sea water is also bactericidal for many nonmarine bacteria, gram-positive organisms being more sensitive than gram-negative forms (Beard and Meadowcroft, 1935, ZoBell, 1936) This was demonstrated by suspending pure or mixed cultures of bacteria from various fresh-water sources in sea water or other mineral solutions and determining the percentage of survival after different periods of time Although gram-negative organisms display resistance to the lethal action of sea water, their viability in this medium varies widely among different species In an investigation employing enteric bacteria, Trawinski (1929) reported survival periods in sea water ranging from 12 hours for *Shigella dysenteriae* to 23 days for *Salmonella enteritidis* Carpenter *et al* (1938) noted that natural sea water killed 80 per cent of the organisms in sewage within half an hour

The bacteriostatic and bactericidal effects of sea water are greater than can be accounted for upon a basis of its salinity or osmotic pressure Not only is natural sea water more bactericidal than synthetic sea water (ZoBell, 1946, ZoBell and Feltham, 1933), but it is also more bactericidal than heat-treated sea water (Kimbayashi and Aida, 1933, Waksman and Hotchkiss, 1937, ZoBell, 1936) De Giaxa (1889) observed that pathogens rapidly perish in raw sea water, although they may survive almost indefinitely in heat-treated sea water Water from the Black Sea was found by Krassilnikov (1938) to be germicidal for terrestrial bacteria until it was boiled He confirmed the observations of Beard and Meadowcroft (1935) and ZoBell (1936) that the bactericidal potency of sea water was decreased but not destroyed by passing it through Berkefeld, Chamberland, Coors, or similar filters

Lacking the properties of bacteriophage, the bactericidal property of sea water is attributed primarily to its content of antibiotic substances produced by microorganisms Credence is lent to this view by the observation that the bactericidal principle occurs in greatest concentration in samples of sea water recently collected from zones of maximum bacterial population The experimental results reported below demonstrate the production of antibiotic substances by several species of microorganisms native to the sea

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EXPERIMENTAL RESULTS

Fifty-eight pure cultures of marine microorganisms were tested for their anti microbial activities   The cultures employed as potential antagonists were taken from a collection previously described by ZoBell and Upham (1944) and were maintained in a medium of the following composition

Difco peptone	5 0 g
Difco yeast extract	1 0 g
(NH₄)₂SO₄	1 0 g
FeSO₄ (NH₄)₂SO₄ 6H₂O	0 1 g
Aged sea water (75 per cent)	1,000 ml

The solution was adjusted to pH 7 4 to 7 6 before autoclaving   When a solid medium was required, 2 0 per cent of agar was added to this solution prior to sterilization

TABLE 1

Antimicrobial behavior of marine microorganisms

ANTAGONISTIC GENUS	SPECIES TESTED	SPECIES INHIBITING FRESH WATER OR TERRESTRIAL ORGANISMS
Bacillus	9	4
Micrococcus	6	3
Actinomyces	2	1
Serratia	1	1
Pseudomonas	19	0
Vibrio	11	0
Flavobacterium	5	0
Achromobacter	4	0
Sarcina	1	0
Totals	58	9

All fresh-water or terrestrial species were tested to ensure their growth in this sea-water medium before subjecting them to the effects of marine antagonists Both antagonistic and test inocula were taken from cultures grown for 48 hours at 27 C

Antimicrobial effects were surveyed in pour plates, each containing 20 ml of nutrient agar and seeded with 0 2 ml of a test culture   After the medium had solidified, the marine species under investigation were streaked over quadrants of the plates, which were then incubated at 27 C   Plates were examined at fre quent intervals over a period of 17 days for growth suppression of the test or ganisms, as evidenced by clearing zones adjacent to the lines of growth of marine antagonists   Table 1 outlines the results noted and summarizes the distribution of antagonistic species among the various genera investigated

Although two antagonistic organisms were gram-negative, the majority species exhibiting inhibitory powers were members of the gram-positive genera *Bacillus* and *Micrococcus*

The susceptibility of the test organisms is described in tables 2, 3, and 4   Of the 11 gram-positive cultures employed, only *Staphylococcus aureus* and *Strepto-*

TABLE 2

Antimicrobial spectrum of marine Bacillus species

TEST SPECIES	INHIBITED BY			
	B borborokoites	B abysseus	B thalassokoites	B submarinus
Bacillus anthracis	+	+	−	−
B megatherium	−	−	−	−
B mycoides	+	+	+	+
B subtilis	−	−	−	−
Corynebacterium pseudodiphthericum	+	−	−	−
Micrococcus roseus	+	+	+	+
Mycobacterium lacticola	−	−	−	−
Proteus vulgaris	−	−	−	−
Salmonella typhimurium	−	−	−	−
Sarcina lutea	+	+	+	+
Shigella paradysenteriae	−	−	−	−
Staphylococcus aureus	−	−	−	−
S citreus	+	+	+	+
Streptococcus faecalis	−	−	−	−

TABLE 3

Antimicrobial spectrum of marine Micrococcus species

TEST SPECIES	INHIBITED BY		
	M maripuniceus	M sedimenteus	M infimus
Bacillus anthracis	+	−	*
B megatherium	−	−	*
B mycoides	−	−	−
B subtilis	−	−	−
Corynebacterium pseudodiphthericum	−	−	*
Micrococcus roseus	−	−	−
Mycobacterium lacticola	+	−	*
Proteus vulgaris	−	−	−
Salmonella typhimurium	−	−	−
Sarcina lutea	+	+	+
Shigella paradysenteriae	−	−	*
Staphylococcus aureus	−	−	−
S citreus	+	−	*
Streptococcus faecalis	−	−	*

* This species not tested

coccus faecalis failed to undergo inhibition by one or more marine species   Three gram-negative species were tested, and none of these was susceptible

An attempt was made to demonstrate the presence of the inhibitory principle in cell-free solutions prepared by filtration of the antagonistic cultures   The

DISCUSSION

Quantitative evidence of the effect of marine microorganisms upon the inhibitory property of sea water has not been obtained   However, a presumptive interrelationship may be inferred from the incidence of antagonistic species   The behavior of antagonistic cultures when passed through germ-proof filters resembles that of sea water, the two undergoing similar decreases in antimicrobial potency   Further evidence for this correlation of activity is observed in a comparison of data reported herein with that testing the effect of sea water upon nonmarine bacteria   Of six organisms shown by Krassilnikov (1938) to be inhibited by unheated sea water and common to both investigations, only *Staphylococcus aureus* failed to demonstrate a bacteriostatic response to marine bacteria It is also significant that the response of test species to inhibition by marine organisms appears to parallel the gram reaction   This is in accordance with the general observation that gram-positive bacteria are more often inhibited by sea water than are gram-negative species

Isolations of specific antibiotics produced by marine bacteria have not been attempted, but it is evident that various species of microorganisms indigenous to the sea elaborate antimicrobial substances   The survey reported here, although very limited in scope, suggests that the marine environment should be considered as a potential source of antibiotics

SUMMARY

Of 58 species of marine microorganisms tested, 9 have demonstrated antibiotic activity against nonmarine forms   The most actively antagonistic marine genera were *Bacillus* and *Micrococcus*

Similarities in the behavior of sea water and of antagonistic marine cultures indicate that the bacteriostatic or bactericidal activity of the former may be at least partially due to an autochthonous flora of antibiotic-producing organisms

It is suggested that the sea may represent a reservoir of microbial antagonists of possible importance

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NOTES

STREPTOMYCIN TOLERANCE OF SAPROPHYTIC AND PATHOGENIC FUNGI

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Relatively little information is available concerning the effect of streptomycin on the growth of pathogenic fungi During the course of an investigation of fungal culture media (reported elsewhere) it became necessary to test the growth of a number of saprophytic and pathogenic fungi on agar containing 30 units of streptomycin sulfate per ml (Winthrop, Cutter) It was observed that growth of the following fungi was found to be unaffected by the concentration of antibiotic employed *Blastomyces dermatitidis* (2 strains), *Blastomyces brasiliensis, Coccidioides immitis, Histoplasma capsulatum, Sporotrichum schenkii, Hormodendrum pedrosoi, Hormodendrum compactum, Phialophora verrucosa, Cryptococcus neoformans, Candida albicans, Candida candida, Microsporum audouini, Microsporum canis, Microsporum gypseum, Trichophyton schoenleinii, Trichophyton violaceum, Trichophyton rubrum, Trichophyton mentagrophytes, Epidermophyton floccosum, Monosporium apiospermum, Geotrichum* sp , *Penicillium expansum, Aspergillus herbariorum, Rhizopus nigricans, Neurospora sitophila, Fusarium, Alternaria, Cladosporium, Mucor mucedo*

Although the fungi listed above may possibly be inhibited by stronger concentrations of streptomycin, their tolerance to 30 units per ml *in vitro* indicates that systemic and cutaneous infections caused by the pathogenic species are not likely to respond well to clinical treatment with streptomycin

NUTRITIONAL STUDIES ON PIRICULARIA ORYZAE[1]

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Piricularia oryzae Cav , the cause of the disease known as "rice blast" which is commonly found in many of the rice-growing areas of the world, varies considerably in quantity and quality of growth on natural media, as shown by Henry and Andersen (1944) This paper is a report of the development of chemically defined ("synthetic") media for *P oryzae* with the aim of producing growth and sporulation comparable to the best natural media and of reducing the degree of variation in conidia production on subculture below that found when the fungus is grown on a natural medium such as rice polish agar

Little is known of the nutritional requirements of *P oryzae* Tochinai and Nakano (1940) reported growth on a synthetic medium containing only NH_4NO_3, $MgSO_4$, xanthine, glucose, and inorganic salts Attempts in this laboratory to confirm their work were unsuccessful

METHODS

A simplified medium (chemically defined except for the presence of purified agar and acid-hydrolyzed "vitamin-free" casein) was prepared for the cultivation of the fungus All ingredients were included which are commonly required by fungi, and several compounds were added which had shown evidence of being beneficial in preliminary experiments (table 1) The usual precautions as to cleanliness of glassware and purity of reagents necessary in nutritional studies were observed

The vitamin-free casein was hydrolyzed with H_2SO_4, which was subsequently nearly neutralized with $Ba(OH)_2$ to remove all but a small amount of sulfate After the precipitate was washed with distilled water, the hydrolyzed casein solution was clarified with charcoal at pH 3 5 to 4 0 until it was nearly colorless The concentrations of hydrolyzed casein reported in the tables were computed on the basis of the amino nitrogen content of the hydrolyzates A bacteriological assay[4] of the medium (table 1) with *Lactobacillus casei* showed that it contained no biotin, no pantothenic acid, and approximately 0 15 μg per ml of nicotinic acid A chemical assay[4] showed less than 0 04 μg per ml of thiamine

Commercial bacteriological agar was washed three times with a mixture of equal parts of pyridine and ethyl alcohol, then with distilled water until no trace

of pyridine could be detected by odor, and finally washed three more times with distilled water   It was dried at 50 C for a minimum of 3 days   All of the growth media reported in this paper were solidified with 2 0 per cent of this purified agar

All cultures were grown in selected 18-by-150-mm pyrex culture tubes in which the media were slanted uniformly   The average area was found to be 9 6 cm², with variations of not more than 0 5 cm² among individual tubes

The stock cultures of P $oryzae^5$ were grown on 2 per cent rice polish, 2 per cent agar slants   Conidia from several 5- to 6-day-old cultures were suspended in sterile distilled water by violent shaking   The suspensions were combined and used for inoculating the media in each experiment   This method produced a low concentration of conidia, but the suspension was relatively free from mycelial fragments and undesirable material from the rice polish agar slant   Ten to 20 thousand conidia suspended in 0 5 ml of sterile distilled water were added to each slant by means of a syringe   The suspension was distributed over the entire surface of the slant by tilting and the excess liquid absorbed on the cotton plug Repeated tests showed that for the size of the cotton plug used, the amount of excess liquid absorbed was so small that no contamination occurred because of the wetting of the plug   Uniform growth occurred over the agar surface, and the variation between replicate tubes was held to a minimum

Four replicates of each medium were inoculated and incubated in the dark for 5 days   In the early experiments, incubation was at 22 to 28 C, in later experiments incubation was at 25 to 27 C   The amount of growth obtained was estimated visually   The degree of sporulation was determined microscopically by counting in a Howard chamber the sample obtained by suspending the conidia in each tube in 10 ml water   The conidia counts given in this paper are averages of four replicate tubes   All results on vitamin requirements were analyzed statistically

RESULTS

Vitamin requirements   The requirements for the B vitamins were determined by adding the following vitamins of the B complex to the basal medium (table 1) thiamine hydrochloride, calcium pantothenate, nicotinic acid, biotin, d-riboflavin, and pyridoxine   To test the effects of these vitamins, they were omitted singly and in groups from a medium containing all of these six vitamins   The omission of nicotinic acid, riboflavin, pyridoxine, and calcium pantothenate had no significant effect on the yield of conidia, however, when either thiamine or biotin was omitted no growth occurred   The results are summarized in table 1   Other experiments showed that neither p-aminobenzoic acid nor a folic acid con centrate⁶ affected the growth or production of conidia of P $oryzae$

Six levels of biotin were added to a medium containing only chemically known ingredients other than agar to determine the optimal biotin level for growth and

⁵ The culture of P $oryzae$ was obtained from Dr E C Tullis, U S D A , Beaumont, Texas
⁶ We wish to thank Dr R J Williams, University of Texas, for the gift of this folic acid concentrate

production of conidia   The results (table 2) indicate that the optimal level lies between 0 001 and 0 01 μg per ml   This experiment confirmed, in a more highly purified medium, the previous findings that no B complex vitamins other than thiamine and biotin are required

An attempt was made to replace biotin with cysteine and pimelic acid, as Eakin and Eakin (1942) have done with *Aspergillus niger*   *P oryzae* did not grow on the basal medium when either cysteine (50 μg per L) or pimelic acid (65 μg per L) or a combination of both was added in place of biotin

TABLE 1

Effect of B complex vitamins on growth and production of conidia by P oryzae

VITAMIN OMITTED FROM MIXTURE*	GROWTH	SPORES IN THOUSANDS/CM²		
		Subculture no		
		1	2	3
None	good	16 7		
Nicotinic acid	good	10 3		
Ca pantothenate	good	11 1		
d Riboflavin	good	14 0		
Thiamine HCl	none	0 0		
Pyridoxine	good	12 2		
Biotin	none	0 0		
None	good	108	430	81
Nicotinic acid ⎫ Ca pantothenate ⎬ d Riboflavin ⎪ Pyridoxine ⎭	good	102	670	203
Rice polish agar control	good	62	81	59

Medium (g/L) sucrose, 5 0, acid-hydrolyzed "vitamin free" casein, 1 0, agar, 20, K HPO₄, 0 5, glycerol, 0 05, oleic acid, 0 05, MgSO₄ 7H₂O, 0 5, CaCO₃, 0 05, Na₂CO₃, 0 05, i inositol, 0 04, guanine, 0 05, xanthine, 0 05, uracil,† 0 1, guanidine HCl, 0 05, CuCl₂, 0 0001, 85% H₂MoO₄, 0 00001, H₃BO₃, 0 0005, MnSO₄, 0 001, ZnCl , 0 0005, Fe(NH₄)₂(SO₄)₂, 0 0005   pH 6 5 ± 0 1

* Vitamin mixture (μg/ml) nicotinic acid, 7 0, Ca-pantothenate, 2 5, d-riboflavin, 2 5, thiamine HCl, 2 0, pyridoxine, 1 0, biotin, 0 01

† Synthesized by T/5 W L Mosby

In order to determine whether *P oryzae* can be continuously cultivated in simplified media, the fungus was carried for 6 successive subcultures on a chemically defined agar medium and the growth and conidia production compared with 6 corresponding transfers on a 2 per cent rice polish, 2 per cent agar medium The degree of variation in sporulation on subculture in the two types of media was analyzed statistically   The results are presented in table 3   It is apparent that a good chemically defined medium supports adequate sporulation with less variation on subculture than does the natural medium

Nitrogen requirements   The requirements of *P oryzae* for amino nitrogen were studied by the omission of each amino acid from a medium containing a mixture of 15 amino acids   The mixture contained glycine, *l*(+)lysine, *dl*-valine,

$l(-)$leucine, d'-isoleucine, dl-threonine, dl-phenylalanine, dl-methionine, dl glutamic acid, dl-aspartic acid, $l(-)$proline, $l(-)$hydroxyproline, $l(+)$arginine, $l(-)$tryptophane, and $l(+)$histidine in concentrations equivalent to their pro

TABLE 2

Determination of optimal biotin level for growth and conidia production by P oryzae

BIOTIN (μG/ML)	SPORE COUNT IN THOUSANDS PER CM^2	VISUAL ESTIMATION OF GROWTH
0 0	0	none
0 00001	0	very slight
0 0001	4 2	poor
0 001	1,220	good
0 01	1,880	good
0 02	1,610	good

Basal medium (g/L)   glucose, 5 0, acid-hydrolyzed "vitamin-free" casein, 1 0, agar, 20, 62% potassium glycerol phosphate, 0 9, MgSO$_4$ 7H$_2$O, 0 5, i-inositol, 0 02, guanine, 0 0066, xanthine, 0 0066, uracil, 0 0066, guanidine HCl, 0 0066, choline Cl, 0 001, thiamine HCl, 0 002, CuCl$_2$, 0 0001, 85% H$_2$MoO$_4$, 0 0001, H$_3$BO$_3$, 0 0005, MnSO$_4$, 0 001, ZnCl$_2$, 0 0005, Fe(NH$_4$)$_2$(SO$_4$)$_2$, 0 0005   pH 6 5 $\pm$ 0 1

TABLE 3

Comparison of variations of conidia production upon subculture of P oryzae on a rice polish medium and a chemically defined medium

SUBCULTURE NO	2% RICE POLISH 2% AGAR MEDIUM CONIDIA IN THOUSANDS/CM^2	CHEMICALLY DEFINED MEDIUM* CONIDIA IN THOUSANDS/CM^2
1	1,700	896
2	771	1,250
3	1,860	615
4	2,720	844
5	760	760
6	292	781
Total	8,100	5,150
Average	1,350	858
Mean deviation between subcultures	66 3%	24 9%

*Medium (g/L)   glucose, 5 0, agar, 20, K$_4$P$_2$O$_7$, 0 5, MgSO$_4$ 7H$_2$O, 0 5, CaCl$_2$, 0 05, Na CO$_3$, 0 05, glycerol, 0 05, thiamine HCl, 0 002, choline Cl, 0 001, biotin, 0 00001, inositol, 0 02, guanine, 0 0066, xanthine, 0 0066, uracil, 0 0066, guanidine HCl, 0 0066, $l(-)$tryptophane, 0 0193 (0 0001 M), dl-glutamic acid, 0 0144 (0 0001 M), $l(-)$leucine, 0 013 (0 0001 M), $l(-)$proline, 0 0117 (0 0001 M), $l(+)$histidine, 0 0154 (0 0001 M), glycine, 0 788 (to raise amino nitrogen to level equivalent to 0 1% casein), CuCl$_2$, 0 0001, 85% H$_2$MoO$_4$, 0 00001, H$_3$BO$_3$, 0 0005, MnSO$_4$, 0 001, ZnCl$_2$, 0 0005, Fe(NH$_4$)$_2$(SO$_4$)$_2$, 0 0005   pH 6 5 $\pm$ 0 1

portions in 0 1 per cent casein   The single omission of each of the amino acids from the medium made little difference in growth or conidia production   In later work it became apparent that any one of several amino acids could function equally well as a nitrogen source, provided a concentration at least equal to the

amino nitrogen of 0 1 per cent casein was used   The results of this work, including 6 successive subcultures, are presented in table 4   It is evident that *P oryzae* can be maintained in subculture in media such as those given in table 4

TABLE 4

Effects of various amino acids and of $(NH_4)_2SO_4$ as nitrogen sources in the continuous cultivation of P oryzae

NITROGEN SOURCE	G/L	SPORES IN THOUSANDS/CM²					
		Subculture no					
		1	2	3	4	5	6
Casein	1 0	310	247	55 3	101	223	111
(NH₄) SO₄*	0 75	4 2	7 0	2 1	2 1	3 5	5 6
Glycine†	0 86	213	334	56 0	98 7	89 6	244
dl Tryptophane Glycine†	0 204 0 7	83 3	134	179	56	104	200
dl Glutamic acid Glycine†	0 147 0 7	101	103	128	125	119	120
dl Leucine Glycine†	0 131 0 7	82 6	94 5	129	62 3	76 3	132
dl-Tryptophane *dl* Glutamic acid *dl* Leucine Glycine†	0 204 0 147 0 131 0 42	43 4	82 6	26 6	22 8	35 0	72 8
l(−)Tryptophane *dl* Glutamic acid *l*(−)Leucine *l*(−)Proline 1(+)Histidine Glycine†	0 0204 0 0147 0 0131 0 0115 0 0155 0 79	120	179	302	82 6	229	252

Medium (g/L)   glucose, 5 0, agar, 20, 62% potassium glycerol phosphate, 0 9, $MgSO_4$ $7H_2O$, 0 001, $CaCl_2$, 0 0005, Na_2CO_3, 0 0005, *i* inositol, 0 02, guanine, 0 0066, xanthine, 0 0066, uracil, 0 0066, guanidine HCl, 0 0066, choline Cl, 0 001, thiamine HCl, 0 002, biotin, 0 00001, $CuCl_2$, 0 0001, 85% H_2MoO_4, 0 00001, H_3BO_3, 0 0005, $MnSO_4$ 0 01, $ZnCl_2$, 0 0005, $Fe(NH_4)_2(SO_4)_2$, 0 0005   pH 6 5 ± 0 1

* Conidia produced in $(NH_4)_2SO_4$ media were morphologically abnormal and failed to germinate   Subcultures in $(NH_4)_2SO_4$ media were made by mycelial transfer

† Glycine was added in each experiment to raise the concentration of amino nitrogen to that of 0 1% casein

Experiments were then undertaken to determine the various sources of organic nitrogen, other than amino acids, which are available to this fungus   Two basal media were used, one containing no organic nitrogen (except that in 0 01 μg per ml biotin and 2 0 μg per ml thiamine HCl) and the other containing 12 5 μg per

ml of total organic nitrogen of which 3 7 μg per ml were amino nitrogen   The compounds to be tested were all added to the basal medium in 0 1 per cent con centration   The results are presented in table 5   Although a large number of widely diverse nitrogenous compounds supported growth and conidia formation,

TABLE 5

Effects of various nitrogen sources on the growth and sporulation of P oryzae

TEST COMPOUND*	"NITROGEN FREE" BASAL MEDIUM†		"NITROGEN FREE" BASAL MEDIUM PLUS MIXTURE OF NITROGENOUS NUTRIENTS‡		
	Growth	Conidia in thousands/cm²	Growth	Conidia in thousands/cm²	
				1	2
Casein	+++++	790	++++	235	20 0
Glycine	++++	113	+++	409	369
Betaine	+	0	+++	83 3	61 6
ε NH₂ caproic acid	+	0	++	31 5	74 2
Guanidine HCl	+	0	+++	83 3	57 4
Urea	++	7 0	+++	110	72 8
Thiourea	0	0	0	0	0 ΅
Nicotinic acid	+	0	++	41 3	46 2
p NH₂-benzoic acid	+	0	++	0	3 5
Uracil	++	7 0	+++	10 5	55 3
Uric acid	+++	14 0	+++	67 9	120
Caffeine (citrated)	+	0	++	78 4	32 2
Triethanolamine	+	0	+++	34 3	63 0
Ethanolamine	+	0	+++	20 3	35 0
Choline chloride	+	0	+++	44 8	36 4
Hydroxylamine	0	0	0	0	0
NH₄Cl	0	0	+		9 8
None	0	0	+		37 1

* All compounds except NH₄Cl were tested at 0 1% concentration   The pH was ad justed to 6 5 ± 0 1 before inoculation of the media   NH₄Cl was tested at 0 06% concen tration, equivalent to 0 1% casein

† "Nitrogen-free" basal medium (g/L)   glucose, 5 0, 62% potassium glycerophos phate, 0 9, agar, 20, MgSO₄ 7H O, 0 1, CaCO₃, 0 05, Na CO₃, 0 05, ι-inositol, 0 02, thia mine HCl, 0 002, biotin, 0 00001, CuCl , 0 0001, 85% H₂MoO₄, 0 00001, H₃BO₃, 0 0005, MnSO₄, 0 001, ZnCl₂, 0 0005, Fe(NH₄)₂(SO₄)₂, 0 0005   pH 6 5 ± 0 1

‡ Mixtures of nitrogenous nutrients (μg/ml in final medium)   guanine, 6 6, xanthine, 6 6, uracil, 6 6, guanidine HCl, 6 6, choline Cl, 1 0

α-amino acids were required for full activity   The amino acid requirement was apparently satisfied by glycine alone

With the medium (table 4) containing the six amino acids (glycine, trypto phane, histidine, leucine, proline, and glutamic acid), choline, inositol, guanine, guanidine, uracil, and xanthine were omitted from the medium singly and as a group to determine whether any of these compounds are essential in an amino acid basal medium   The results (table 6) indicate that none of these compounds are essential for growth or conidia production during a period of 4 subcultures

Some of the compounds named may be beneficial in stabilizing the growth and conidia production of the organism

Some of the media were altered for use in submerged cultures by reducing the content of agar to 0 1 per cent   Heavy growth was observed, but conidia were not formed in liquid cultures incubated in aerated bottles   No attempts were made to induce conidia formation in submerged cultures by nutritional alterations   A shaking machine for aeration by agitation was not available during this work, its use might prove valuable in producing submerged sporulated cultures

TABLE 6

Effects of certain accessory growth factors on growth and production of conidia by P oryzae

COMPOUND OMITTED FROM MEDIUM	SPORES IN THOUSANDS/cm^2			
	Subculture no			
	1	2	3	4
None	222	1,420	362	998
Choline Cl	78 4	778	1,350	511
Inositol	81 2	491	314	330
Guanidine HCl	109	312	167	360
Guanine	95 2	225	1,450	750
Xanthine	104	557	1,130	213
Uracil	137	878	918	692
Choline Cl Inositol Guanine Xanthine Uracil Guanidine HCl	146	347	1,280	943

Medium Same as given in table 4 including the six amino acids listed together   The compounds named above were used in the concentrations given in table 4

Conidia of *P oryzae* produced in chemically defined media have shown 97 to 99 per cent germination[7] and have been found as infective[8] for the rice plant in the greenhouse as conidia produced on natural media

DISCUSSION

On the basis of these studies, the nutritional requirements of *P oryzae* appear to be relatively nonspecific except with regard to the vitamins, thiamine and biotin being the only ones required   This fungus requires organically combined nitrogen (preferably α-amino acids), but a large number of compounds in which the nitrogen exists in amino, cyclic, imino, or quaternary combination will support growth and conidia formation   No complete investigations were made of the essentiality of some of the other components of the medium, especially the inorganic ions

[7] We wish to thank Capt J W Marek, AUS, for performing the germination experiments
[8] We wish to thank S/Sgt T L Morgan, AUS, for performing the infectivity experiments

Although the fungus grew more uniformly on the chemically defined media than on natural media, such as rice polish agar, the degree of variation was considerable with all media   The variability of the quantity of growth in replicates prevents accurate evaluation of nutrients the effects of which are quantitatively of a low order   Perhaps further nutritional investigations would lead to greater uniformity, especially if submerged dispersed growth in liquid media could be used in place of surface growth   Although the production of conidia was less variable on an adequate chemically defined medium than on a natural medium (table 3), when certain of the pure nutrient compounds were omitted from chemically defined media to determine their essentiality (tables 4 and 6) the resulting cultures were sometimes as variable as those on rice polish agar

SUMMARY

Thiamine (2 μg per ml or less) and biotin (0 01 μg per ml) are required for growth and conidia formation by *Piricularia oryzae*   Other B complex vitamins are not required

P oryzae requires organically combined nitrogen, preferably α-amino acids, but can use many types of organic compounds in which the nitrogen exists in amino, imino, cyclic, or quaternary combination

P oryzae can be maintained successfully in subculture on chemically defined media, the degree of variation and the yields, viability, and the degree of germination and infectivity of conidia comparing favorably with cultures grown on natural media

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THE TUBERCULOSTATIC ACTION OF PARA-AMINOSALICYLIC ACID[1]

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Bernheim (1941, 1942) reported that sodium benzoate and sodium salicylate increased the oxygen uptake of tubercle bacilli Lehman (1946a) reported that the respiration of virulent tubercle bacilli was stimulated by sodium salicylate but that the respiration of avirulent tubercle bacilli was not On the basis of this work Lehman (1946b), attempting to find a substance which would be bacteriostatic to tubercle bacilli, tested more than 50 derivatives of benzoic acid for their bacteriostatic activity The most active substance found was *para*-aminosalicylic acid (4-amino-2-hydroxybenzoic acid—PAS) This substance was bacteriostatic in a concentration of 0 15 mg per 100 ml for the BCG strain of the bovine type of *Mycobacterium tuberculosis* and, according to Lehman, exerted a favorable effect on clinical tuberculosis In a more recent communication, Lehman (1946c) amplified his earlier work and apparently observed some retardation of experimental tuberculosis of guinea pigs after 7 days' treatment with PAS

Youmans (1946), in a preliminary communication, reported that PAS was highly bacteriostatic for 12 virulent strains of human type tubercle bacilli and exerted a suppressive action on experimental tuberculosis of mice

The present paper details further work on the tuberculostatic activity of *p*-aminosalicylic acid both *in vitro* and *in vivo*

METHODS

The tuberculostatic activity of PAS[2] was tested *in vitro* by the method described previously (Youmans, 1944, Youmans and Doub, 1946) by determining the least amount which would completely inhibit the subsurface growth of 0 01 mg of tubercle bacilli per ml of synthetic medium These tuberculostatic tests were also done in synthetic medium to which was added enough bovine serum to make a final concentration of 10 per cent The effect of sodium salicylate and *para*-aminobenzoic acid (PABA) on the bacteriostatic power of PAS was tested by incorporating the former substances in media in which dilutions of PAS were prepared The test materials were incubated at 37 C, and the results were read at the end of 14 days The human type strains of tubercle bacilli tested had been isolated from patients with tuberculosis within the preceding

year and a half, with the exception of the standard H37Rv strain   In addition, one stock bovine type strain and one avian type, as well as the avirulent rapidly growing strain no 607, were used   Six of the human strains were streptomycin resistant (Williston and Youmans, 1947)

The effect of PAS *in vivo* was determined by infecting mice[3] intravenously with 0 1 mg of the H37Rv strain as previously described (Youmans and Mc Carter, 1946, Youmans and Williston, 1946)   PAS was incorporated in the desired concentration in the mouse diet which consisted of a powdered food obtained by grinding Rockland Farms complete mouse ration pellets and passing them through a sieve in order to eliminate coarse particles   PAS was used in these mouse experiments in the form of both the hydrochloride and the free base, with the latter compound one mole of sodium bicarbonate was added for each mole of PAS   The mice were fed the diets containing PAS for 28 days, starting the day before they were infected with tubercle bacilli

In untreated control and corresponding treated series, animals remaining alive at the end of 28 days were sacrificed by exposure to ether vapor, weighed, and eviscerated   At that time, random tissue specimens were removed and cultured on Herrold's glycerol egg yolk agar and a variety of other media designed to grow most types of organisms   The viscera were then fixed *in toto* in 3 7 per cent formaldehyde solution   Subsequently they were dissected and only the larger parenchymatous organs, i e , lungs, livers, kidneys, and spleens, were retained These were re-examined after fixation, and an evaluation of the extent of the lesions was made on the following basis zero (0), apparently normal, plus or minus (±), no definite lesions but a questionable deviation from normal, one plus sign (+), less than 10 per cent involvement, two plus signs (++), from 10 to 25 per cent involvement, three plus signs (+++), 25 to 50 per cent involvement, and four plus signs (++++), 50 to 75 per cent, or possibly more, of the organ composed of grossly pathologic tissue   Notation was also made of the predominant type of lesion, tuberculoid or patchy, present in the lungs   Because previous experience in this laboratory had indicated a high degree of constancy in the lesions produced by this method, only sample tissues were selected for sectioning from animals in which the greatest or least pathologic change seemed probable   These tissues were dehydrated and embedded in paraffin according to the usual technique, and sections were cut at five microns Each was stained with hemotoxylin and eosin and by the Ziehl-Neelsen carbol fuchsin technique for acid-fast organisms   The extent of microscopic involvement was tabulated on the following basis zero (0), apparently normal, on plus sign (+), less than 10 per cent of the organ involved, two plus signs (++) 10 to 25 per cent replaced, three plus signs (+++), 25 to 50 per cent involve and four plus signs (++++), 50 to 75 per cent, or possibly more, of the org replaced by pathologic tissue   Notation was made of the predominant type lesion present, necrotic-exudative or proliferative   Sections stained for acid f bacilli and studied by oil immersion were graded on the following basis zero (no acid-fast bacilli seen, one plus sign (+), a few single, or clumps of 4 -

intra- or extra-cellular organisms observed in occasional fields, two plus signs
(++), moderate number of single organisms or groups of 4 or 5, observed intra-
or extra-cellularly in about half of the fields, three plus signs (+++), solitary
bacilli or clumps of intra- or extra-cellular organisms seen in more than half of
the fields, and four plus signs (++++), single organisms, clumps, and large
masses of bacilli found within and outside cells in more than half the fields

Experiments were also conducted in which both streptomycin and PAS were
administered to mice infected with tubercle bacilli   In these experiments the

TABLE 1

Bacteriostatic effect of p-amino salicylic acid on streptomycin sensitive and streptomycin-resistant strains of tubercle bacilli

STRAIN NO	TYPE	CONCENTRATION IN MG PER 100 ML WHICH COMPLETELY INHIBITED GROWTH	
		Without plasma	With plasma
H37Rv	Human	0 078	0 156
H37RvR*	Human	0 039	0 039
100	Human	0 078	
100R	Human	0 019	
23	Human	0 039	0 156
23R	Human	0 039	0 078
24	Human	0 019	0 039
24R	Human	0 019	0 019
69	Human	0 019	0 039
69R	Human	0 039	0 078
97	Human	0 0095	0 019
97R	Human	0 019	0 019
111	Human	0 156	
1	Human	0 078	0 156
11	Human	0 039	0 039
12	Human	0 078	0 078
18	Human	0 019	0 019
48	Bovine	No growth	0 039
37	Avian	0 625	0 625
607	?	>100 0	

* R indicates a streptomycin-resistant strain

PAS was administered in the diet as before, whereas streptomycin in distilled
water was given in 2 daily subcutaneous injections of 0 2 ml each, 8 hours apart

RESULTS

Table 1 shows the *in vitro* bacteriostatic effect of PAS on the strains of tubercle
bacilli employed   All of the strains except one were inhibited by very low con-
centrations of PAS   The human strains appeared to be approximately equally
sensitive to the bacteriostatic activity of PAS, and furthermore this activity was
not markedly affected by the presence of 10 per cent bovine plasma   There was
no significant difference between the results obtained with the streptomycin-
sensitive and resistant strains   The one bovine strain appeared to be as sus-

ceptible as the human strains, whereas the one avian strain seemed to be slightly more resistant to the bacteriostatic activity of PAS   The rapidly growing avirulent strain no 607, however, was highly resistant to the bacteriostatic activity of PAS, growth occurring even in a concentration of 100 mg per cent

It should also be noted that the bacteriostatic activity of PAS for human type tubercle bacilli was of approximately the same order as that of streptomycin, since in most cases less than 1 microgram per ml of medium completely inhibited growth

Table 2 shows the effect of the number of organisms in the inoculum on the *in vitro* bacteriostatic activity of PAS   The bacteriostatic activity of this compound, as has been observed with the sulfonamides and the sulfones, is markedly affected by the number of organisms present   However, even with inocula which in this experiment gave growth in a concentration of 10 mg per cent there was still partial bacteriostatic activity in a concentration of 0 156 mg per cent, as determined by comparing the growth in these tubes with the control

TABLE 2

The effect of the number of tubercle bacilli (H37Rv) upon the bacteriostatic activity of PAS

NO OF TUBERCLE BACILLI IN MG PER ML OF MEDIUM	CONCENTRATION OF PAS										
	10 0	5 0	2 5	1 25	0 625	0 312	0 156	0 078	0 039	0 019	Controls
0 01	—	—	—	—	—	—	—	—	—	S	G
0 02	—	—	—	—	—	—	—	—	S	S	G
0 03	—	—	—	—	—	—	S	S	M	G	G
0 04	—	—	—	S	S	S	M	M	G	G	G
0 05	S	S	S	S	S	M	M	G	G	G	G

— = no growth, S = slight growth, M = moderate growth, G = good growth

When PAS was tested in a medium containing 0 1 mg PABA per 100 ml, the bacteriostatic activity was reduced to one-sixteenth of its former value   Although this reduction of bacteriostatic activity is not great, it possibly indicates that the bacteriostatic action is, at least in part, due to anti-PABA action

If the activity of PAS is due to its antisalicylate effect, as implied by Lehman (1946b), one would expect sodium salicylate to have some anti-PAS action However, the bacteriostatic activity of PAS was not influenced by the presence of sodium salicylate in the medium in concentrations of 0 1 and 1 0 mg per 100 ml   Lehman (1947) also failed to observe any interference by PAS with th stimulation of respiration by salicylates

The combined bacteriostatic activity of PAS and streptomycin *in vitro* also tested   In all cases, however, the degree of bacteriostasis was no grea than the sum of the individual activities of the two substances

Table 3 shows the *in vitro* bacteriostatic activity of 13 derivatives of PAS salicylic acid [4]   In all cases the bacteriostatic activity is markedly less than of PAS

[4] These compounds prepared by Leonard Doub and Dr L L Bambas, Research I tories, Parke, Davis & Company, Detroit, Michigan

Referring to table 4, it will be noted that inclusion of a 2 per cent concentration of *para*-aminosalicylic acid in the diet prolonged the average survival time of mice infected with 0 1 mg of H37Rv to 27 9 days as compared with the average survival time of 20 2 days for the control series   Inasmuch as all the treated animals but two survived the time limit of the experiment, and one of these was accidentally killed, it is probable that the actual differential is greater than that apparent   Although the treated animals did not experience the expected weight gain for their age (about 5 to 10 grams in 4 weeks), the average final weight was about equal to that recorded initially   This compares favorably with the average weight loss of 5 8 grams for the control animals   Again it will be noted that the treated animals averaged 1 7+ gross pulmonary involvement

TABLE 3

Bacteriostatic activity of derivatives of p-aminosalicylic acid and derivatives of salicylic acid on M tuberculosis (H37Rv)

COMPOUND	LEAST AMOUNT IN MG/100 ML WHICH COMPLETELY INHIBITS GROWTH OF M H37Rv
p-Aminosalicylic acid	0 039–0 078
5 Amino 2 hydroxybenzoic acid	>10 0
4 Amino-2 hydroxybenzamide	10 0
Ethyl-4 amino 2 hydroxy benzoate	2 5
4 Amino 2-methoxybenzoic acid	>10 0
4 Acetylamino 2 hydroxybenzoic acid	10 0
m Aminophenol	>10 0
Salicylic acid	10 0
2 Methoxybenzoic acid	>10 0
2 Hydroxybenzyl alcohol	10 0
3 Hydroxybenzoic acid	>10 0
4-Amino-2 hydroxybenzene sulfonic acid	>10 0
Thiosalicylic acid	5 0
4 Amino-2 hydroxybenzene arsonic acid	5 0

as compared with the average of 3 8+ in the control series   Qualitative histopathologic changes in the treated and untreated groups were related in reciprocal manner   Thus the majority of the control animals exhibited greatly enlarged, tubercle-bearing lungs, the unit lesion of which was of necrotic-exudative character with large numbers of bacilli   Conversely, the majority of the treated animals' lungs were smaller and were the site of proliferative changes with fewer mycobacteria present   These findings suggest a lack of general tissue toxicity or depression of the defensive mechanism   The predominance of the proliferative lesions at prolonged survival times, hence longer evolution times for the unit lesions, implies a depressive influence exerted on the bacteria or a stimulating influence on resistance   In view of the *in vitro* effectiveness of this compound it seems logical to assume that at least the greater portion of this effect was exerted upon the bacteria directly

Increasing the concentration of the drug to 4 per cent in the diet resulted in a

reduction of average survival time to 14 4 days and the disproportionately great average weight loss of 6 3 grams for this short survival period   The histopathology of the unit lesions was in keeping with the short survival time   These data indicate a toxic effect exerted by the drug on the body tissues in general

When PAS was employed in a 1 per cent concentration in the diet, survival time was prolonged to the limits of the experiment, whereas the average control animal survived 20 8 days   Here again, the actual differential is probably greater than that apparent   The treated animals experienced an average weight gain of 1 2 grams as compared with the average loss of 5 1 grams for the control series   Whereas the extent of gross pulmonary change in the treated animals averaged 2 9+ as compared with the average of 3 8+ for the control series, the

TABLE 4

Effect of p-aminosalicylic acid on experimental tuberculosis of white mice

NUMBER OF MICE	PER CENT PAS IN DIET	NUMBER DEAD	PER CENT MORTALITY	AVERAGE SURVIVAL TIME	AVERAGE WEIGHT LOSS OR GAIN	AVERAGE AMOUNT GROSS PULMONARY TUBERCULOSIS
		p-Aminosalicyclic acid hydrochloride				
20	0 0	17	85 0	20 2	−5 8	3 8+
19*	2 0	1	5 26	27 9	+0 4	1 7+
20	4 0	13	65 0	14 4	−6 3	1 2+
20	0 0	15	75 0	20 8	−5 1	3 9+
20	1 0	0	0 0	28 0	+1 2	2 9+
		p Aminosalicylic acid (free base)				
15	0 0	12	80 0	23 6	−5 2	3 8+
15	1 0	0	0 0	28 0	+1 0	2 4+
15	2 0	0	0 0	28 0	−1 2	1 6+
15	4 0	12	80 0	13 2		2 4+

* One mouse killed accidentally and not included

proliferative histopathologic pattern, with low bacterial concentrations, predominated in the former group   These data follow the same pattern as those determined with the 2 per cent concentration   Since the control data for both these series are closely parallel, comparison of the two treated groups seems va.. It is apparent that the 1 per cent concentration permitted a somewhat m' extensive pulmonary change, still of chiefly proliferative pattern, and a great weight gain   This seeming paradox might be construed as evidence that 2 per cent concentration was slightly toxic, even though evidence of its t was not previously adduced, and that the 1 per cent concentration exerted s. what less depressive action on the bacteria

Since it was felt that the toxicity observed in these experiments might be ' the acidity of the compound when used in the form of the hydrochloride next experiment was done employing PAS in the form of the free base, to

was added sodium bicarbonate   The results, however, were in every respect similar to the first experiment both as to survival time and the extent of the gross and microscopic lesions in the lungs (table 4)   Tubercle bacilli were recovered by culture from both control and PAS-treated animals, but in fewer numbers from the latter animals   No other types of bacteria were isolated

From these results it is apparent that PAS is moderately effective in suppressing experimental pulmonary tuberculosis of mice but is toxic in these animals in concentrations of over 1 per cent   Lehman (1947) states that mice tolerate well concentrations of this drug up to 5 per cent   The reason for this discrepancy in our results is not apparent unless the diets which were different in Lehman's experiments than in ours might have influenced the toxic reactions obtained

Previous experience in this laboratory with similarly conducted experiments (Youmans and McCarter, 1946) has demonstrated the effectiveness of streptomycin therapy in prolonging the survival time and reducing the weight loss of the experimental animals, as well as in the reduction of the extent of the pathologic change in the organs and the concentration of bacteria in the unit lesions   Further experience with that antibiotic agent has demonstrated a correlated qualitative change in that the pulmonary lesions of the treated animals were predominantly of the proliferative type, whereas those of the control groups are principally of the necrotic-exudative type   In the experience of the present writers, these favorable patterns are somewhat more impressive when streptomycin is employed in optimum subcutaneous dosage than when *para*-aminosalicylic acid is added to the diet in either 1 or 2 per cent concentration

Preliminary work, however, indicates that tuberculous mice treated with both streptomycin and PAS show a therapeutic response greater than that observed with either substance alone   As the effect appears to be no more than additive, the implications in the treatment of clinical tuberculosis are obvious   These results will be reported in detail in a subsequent communication

<div align="center">SUMMARY</div>

Para-aminosalicylic acid was found to be highly bacteriostatic *in vitro* for virulent human type tubercle bacilli, and this activity was not appreciably affected by the presence of serum in the medium   The bacteriostatic activity was partially reversed by *para*-aminobenzoic acid but was not reversed by sodium salicylate   Furthermore, the bacteriostatic activity of this compound (PAS) was inversely proportional to the number of organisms present in the medium   One avirulent, rapidly growing, acid-fast organism, no 607, was not inhibited by 100 mg per cent *para*-aminosalicylic acid

Thirteen derivatives of PAS and salicylic acid were found to be much less tuberculostatic than PAS

Experimental tuberculosis of mice was found to be suppressed by *para*-aminosalicylic acid when it was administered in the diet in 1 and 2 per cent concentrations, both when the drug was given in the form of the hydrochloride and in the form of free base   Under the conditions of the experiment, 4 per cent *para*-

aminosalicylic acid when administered to mice was highly toxic, whereas 2 per cent was slightly toxic

PAS and streptomycin when administered to mice simultaneously appeared to exert a suppressive effect on the tuberculous process greater than that of either substance alone

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THE BACTERIOSTATIC ACTIVITY OF CERIUM, LANTHANUM, AND THALLIUM

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The salts of cerium, lanthanum, and thallium have long been known to possess bacteriostatic properties in low concentrations, but there is little information in the literature concerning the variability of their toxicity toward different species of microorganisms A report of the concentrations of the salts limiting development of various types of bacteria may have importance in relation to their possible use as bacteriostatic or bactericidal agents

Bokorny (1894) found cerium compounds relatively much more toxic for bacteria than for algae Hebert (1907) reported that cerium and lanthanum sulfates in concentrations of 5 to 10 grams per liter showed little toxicity toward *Aspergillus niger* and yeast Sartory and Bailly (1922) reported that 0 2 per cent lanthanum sulfate depressed the growth of *Aspergillus fumigatus* in Raulin's solution and practically inhibited spore formation Frouin (1912) observed that 0 005 grams of lanthanum sulfate per 100 ml of medium stimulated the growth of the tubercle bacillus but that higher concentrations were toxic Frouin and Roudski (1914) studied the toxicity of lanthanum and thorium for the cholera and dysentery organisms

Other investigators who reported bacteriostatic or lethal effects of salts of the rare earth group include Drossback (1897), Brooks (1921), Grenet and Drouin (1927), Zirpolo (1924), Frouin (1920), Simonini (1914), Doerr (1920), Eisenberg (1918), and Hotchkiss (1923) A general review of the earlier literature on this subject is found in Buchanan and Fulmer (1930) McKenzie (1941) employed thallium acetate in a medium recommended for the enrichment of the streptococci causing mastitis The effect of cerium on enzyme activity was reported by Gould (1936) Olszewski (1932) observed no significant reduction in the bacteria of river water when 1 ppm of cerous or ceric chloride or ceric sulfate was employed Richards (1932) reported thallium to be a growth stimulant for yeast

The present paper reports a further investigation of the bacteriostatic activity of the salts of cerium, lanthanum, and thallium

METHODS

Thirty-nine species of bacteria, representing 16 different genera, were employed in this study Also, 35 species of fungi, comprising 18 genera, were used in a limited comparison of the mycostatic and bacteriostatic effects of the compounds The salts used were cerium chloride ($CeCl_3$, cp, E H Sargent), cerium nitrate ($Ce(NO_3)_3$ $6H_2O$, cp, General Chemical Co), anhydrous ceric sulfate ($Ce(SO_4)_2$, G F Smith Chemical Co), lanthanum chloride (cp, E H Sargent), and thallium nitrate (E H Sargent)

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Other chemicals were used in some of the tests in order to determine their effect on the toxicity of the test substances   These chemicals included the sulfates and chlorides of sodium, magnesium, barium, and ammonium, and the chlorides of calcium and lithium   Stock solutions of the salts were made and the amount required for each test medium was removed by pipette   The tests were made on petri plates of solidified agar containing the specified amounts of the salts   The basal medium consisted of 1 per cent Difco peptone and 1 5 per cent Difco agar in distilled water   Inoculations of the agar plates were made by means of a 1½-mm nichrome wire loop using a 24-hour broth culture   Radial streak inoculations were made, using 8 cultures to each plate   Incubation was at 37 C except for those soil and water forms which grow better at a lower temperature and were incubated at room temperature (22 to 27 C)   Observations and records were made after incubation for 1, 2, 3, and 5 days

<div align="center">RESULTS</div>

The toxicity of cerium salts for bacteria   The bacteriostatic action of three cerium salts, the trivalent cerium chloride and cerium nitrate and the tetravalent ceric sulfate, was determined against 40 different species   The results are presented in table 1   The chloride was found to be definitely less toxic than either the sulfate or nitrate of cerium   No significant difference in toxicity between the sulfate and nitrate of cerium was noted

The reaction of the media was not adjusted after the addition of the cerium salts, and the pH values were found to vary as follows for cerium chloride agar, 6 3 at 0 0002 M concentration to 5 95 at 0 0014 M concentration, for cerium nitrate, pH 5 8 at 0 0001 M to 5 65 at 0 0009 M, and for cerium sulfate, pH 6 7 at 0 0002 M to 5 4 at 0 0008 M

The toxicity of cerium nitrate and cerium chloride varied relatively little among the different species of bacteria tested, and even fewer variations were observed with cerium sulfate   Of the bacterial species tried, *Aerobacter aerogenes, Aerobacter cloacae, Salmonella aertrycke,* and *Achromobacter lipolyticum* were most tolerant of cerium   The *Torula rosea* culture proved to be far more resistant to the cerium compounds than the most resistant bacteria

The effect of pH and the presence of other salts on the toxicity of cerium compounds   In order to determine the effect of pH on cerium toxicity, the media were prepared using cerium nitrate in concentrations varying from 0 0003 M to 0 0007 M, then adjusted to pH 6 0 and 8 0   The results obtained after 2 days' incubation are presented in table 2   It was observed that at pH 8 0 all cultures except *Staphylococcus albus* developed without hindrance in 0 0007 M cerium nitrate whereas at pH 6 0 a considerable number of cultures failed to grow in the 0 000 M concentration of cerium

The effect of other salts on the bacteriostatic activity of cerium compounds   T effect of various salts which are sometimes used in culture media on the b teriostatic activity of cerium compounds was determined by adding the c⁻ separately to the cerium-containing media and observing for bacterial gro after inoculations   It was found that sodium chloride in concentrations

TABLE 1

The toxicity of various cerium compounds for certain bacteria

Incubation for 2 days

CULTURE	MOLECULAR CONCENTRATION					
	CeCl₂		Ce(NO₃)₃		Ce(SO₄)₂	
	A	B	A	B	A	B
Salmonella paratyphi	0 0006	0 0008	0 0004	0 0005	0 0004	0 0006
Salmonella pullorum	0 0006	0 0008	0 0004	0 0005	0 0004	0 0006
Salmonella schottmuelleri	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Salmonella enteritidis	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Salmonella aertrycke	0 0008	0 0010	0 0004	0 0005	0 0006	0 0008
Salmonella gallinarum	0 0006	0 0008	0 0004	0 0005	0 0004	0 0006
Salmonella suipestifer	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Eberthella typhosa	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Shigella sonnei	0 0008	0 0010	0 0005	0 0006	0 0004	0 0006
Shigella dysenteriae	0 0008	0 0010	0 0003	0 0004	0 0004	0 0006
Aerobacter aerogenes	0 0008	0 0010	0 0005	0 0006	0 0006	0 0008
Aerobacter cloacae	†	†	0 0005	0 0006	0 0006	0 0008
Escherichia coli	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Escherichia communior	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Escherichia acidilactici	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Citrobacter intermedium	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Alcaligenes faecalis	0 0010	0 0012	0 0004	0 0005	0 0004	0 0006
Proteus vulgaris	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Pseudomonas aeruginosa	0 0004	0 0006	0 0003	0 0004	0 0004	0 0006
Pseudomonas ovalis	0 0006	0 0008	0 0003	0 0004	0 0004	0 0006
Pseudomonas graveolens	0 0004	0 0006	0 0001	0 0002	0 0004	0 0006
Pseudomonas syncyanea	0 0006	0 0008	0 0003	0 0004	0 0004	0 0006
Pseudomonas mucedolens	0 0004	0 0006	0 0001	0 0002	0 0004	0 0006
Flavobacterium sauveolens	0 0010	0 0012	0 0005	0 0006	0 0004	0 0006
Achromobacter lipolyticum	0 0010	0 0012	0 0006	0 0007	0 0006	0 0008
Serratia marcescens	0 0010	0 0012	0 0006	0 0007	0 0004	0 0006
Bacillus subtilis	0 0010	0 0012	0 0006	0 0007	0 0004	0 0006
Bacillus mesentericus	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Bacillus mycoides	0 0008	0 0010	0 0005	0 0006	0 0004	0 0006
Bacillus fusiformis	0 0008	0 0010	0 0004	0 0005	0 0004	0 0006
Bacillus metiens	0 0008	0 0010	0 0005	0 0006	0 0004	0 0006
Staphylococcus candidus	0 0008	0 0010	0 0005	0 0006	0 0004	0 0006
Staphylococcus flavus	0 0008	0 0010	0 0003	0 0004	0 0004	0 0006
Staphylococcus aureus	0 0008	0 0010	0 0007	0 0008	0 0004	0 0006
Staphylococcus albus	0 0010	0 0012	0 0004	0 0005	0 0004	0 0006
Sarcina lutea	0 0006	0 0008	0 0004	0 0005	0 0004	0 0006
Sarcina conjunctivae	0 0006	0 0008	0 0007	0 0008	0 0004	0 0006
Rhodococcus agilis	0 0006	0 0008	0 0003	0 0004	0 0004	0 0006
Rhodococcus rosaceous	0 0006	0 0008	0 0003	0 0004	0 0004	0 0006
Torula rosea	0 0012	*	0 0009	*	0 0008	*

A = concentration permitting growth in 2 days

B = concentration inhibiting growth in 2 days

* Inhibiting concentration was not determined

† Concentration less than 0003 M was not employed

TABLE 2
The effect of pH on the toxicity of $Ce(NO_3)$ for certain bacteria

CULTURE	MOLECULAR CONCENTRATION			
	pH 6		pH 8	
	A	B	A	B
S paratyphi	0 0003	0 0004	0 0007	*
S pullorum	0 0003	0 0004	0 0007	*
S schottmuelleri	0 0005	0 0007	0 0007	*
S enteritidis	0 0004	0 0005	0 0007	*
S aertrycke	0 0004	0 0005	0 0007	*
S gallinarum	0 0004	0 0005	0 0007	*
S suipestifer	0 0005	0 0006	0 0007	*
E typhosa	0 0004	0 0005	0 0007	*
S sonnei	†	0 0003	0 0007	*
S dysenteriae	0 0003	0 0004	0 0007	*
A aerogenes	0 0007	*	0 0007	*
A cloacae	0 0006	0 0007	0 0007	*
E coli	0 0004	0 0005	0 0007	*
E communior	0 0004	0 0005	0 0007	*
E acidilactici	0 0003	0 0004	0 0007	*
C intermedium	0 0006	0 0007	0 0007	*
A faecalis	0 0003	0 0004	0 0007	*
P vulgaris	0 0003	0 0004	0 0007	*
P aeruginosa	†	0 0003	0 0007	*
P ovalis	†	0 0003	0 0007	*
P graveolens	†	0 0003	0 0007	*
P syncyanea	†	0 0003	0 0007	*
P mucedolens	†	0 0003	0 0007	*
F suaveolens	0 0007	*	0 0007	*
A lipolyticum	0 0007	*	0 0007	*
S marcescens	0 0007	*	0 0007	*
B subtilis	†	0 0003	0 0007	*
B mesentericus	0 0003	0 0004	0 0007	*
B mycoides	†	0 0003	0 0007	*
B fusiformis	†	0 0003	0 0007	*
B meliens	†	0 0003	0 0007	*
S aureus	0 0006	0 0007	0 0007	*
S candidus	0 0004	0 0005	0 0007	*
S albus	0 0003	0 0004	0 0004	0 0005
S flava	0 0003	0 0004	0 0007	*
S lutea	†	0 0003	0 0007	*
S conjunctivae	†	0 0003	0 0007	*
R agilis	0 0003	0 0004	0 0007	*
R rosaceous	†	0 0003	0 0007	*
Torula rosea	0 0007	*	0 0007	*

A = concentration permitting growth in 2 days
B = concentration inhibiting growth in 2 days
* Concentration greater than 0 0007 M was not employed
† Concentration less than 0 0003 M was not employed

TABLE 3

The effect of 0 4 M of NaCl and 0 1 M Na SO₄ on toxicity of Ce(SO₄)₂

CULTURE	Ce(SO₄)₂ CONC (M)	CONTROLS FOR			Ce(SO₄)₂ PLUS	
		Ce(SO₄)	NaCl	Na₂SO₄	NaCl	Na₂SO₄
		Vigor of growth incubation for 2 days				
S paratyphi	0 0006	—	++	++	—	+
S pullorum	0 0006	—	++	++	—	++
S schottmuelleri	0 0008	—	+++	+++	—	++
S enteritidis	0 0008	—	++	++	—	+
S aertrycke	0 0008	+	++	++	—	+
S gallinarum	0 0004	+	++	++	++	+
S suipestifer	0 0008	—	++	++	—	+
E typhosa	0 0006	—	+++	+++	—	++
S sonnei	0 0006	—	++	++	—	+
S dysenteriae	0 0004	++	++	++	+	+
A aerogenes	0 0008	+	++	++	—	++
A cloacae	0 0008	++	+++	++	—	++
E coli	0 0006	—	+++	++	—	+
E communior	0 0008	—	+++	+++	—	+
C intermedium	0 0006	—	++	++	—	+
A faecalis	0 0006	—	+++	++	—	+
P vulgaris	0 0006	—	++	++	—	+
P aeruginosa	0 0004	++	++	+++	—	++
P ovalis	0 0004	++	++	++	+	++
P graveolens	0 0004	++	++	++	—	+
P syncyanea	0 0004	+	+++	+++	—	++
P mucedolens	0 0004	+	++	++	—	+
F suaveolens	0 0006	—	++	++	—	++
A lipolyticum	0 0008	+	++	++	+	+
S marcescens	0 0006	+	++	++	+	++
B subtilis	0 0006	+	++	+++	—	++
B mesentericus	0 0008	—	++	+++	—	+
B mycoides	0 0004	++	++	+++	+	++
B fusiformis	0 0004	++	++	+++	++	++
B meliens	0 0004	++	++	++	++	++
S aureus	0 0004	++	++	++	++	++
S candidus	0 0006	—	++	++	—	+
S albus	0 0004	++	++	++	+	++
S flavus	0 0004	++	++	++	+	+
S lutea	0 0004	+	++	++	—	+
S conjunctivae	0 0004	++	++	++	+	++
R agilis	0 0004	+	++	++	+	+
R rosaceous	0 0004	+	++	++	+	+
Torula rosea	0 0004	++	++	++	++	++

(—) = complete inhibition of growth
(+) = moderate growth
(++) = good growth
(+++) = growth better than on nutrient agar control

TABLE 4

The bacteriostatic activity of lanthanum chloride and thallium nitrate

	MOLECULAR CONCENTRATION			
ORGANISM	Permitting growth		Preventing growth	
	LaCl₃	TlNO₃	LaCl₃	TlNO₃
S paratyphi	0 0002	0 0006	0 0004	0 0007
S pullorum	0 0002	0 0006	0 0004	0 0007
S schottmuelleri	0 0004	0 0007	0 0006	0 0008
S enteritidis	0 0004	0 0007	0 0006	0 0008
S aertrycke	0 0004	0 0007	0 0006	0 0008
S gallinarum	0 0004	0 0006	0 0006	0 0007
S suipestifer	0 0004	0 0006	0 0006	0 0007
E typhosa	0 0004	0 0006	0 0006	0 0007
S conjunctivae	0 0006	0 0008	0 0008	0 0010
S sonnei	0 0004	0 0007	0 0006	0 0008
S dysenteriae	0 0004	0 0005	0 0006	0 0007
A aerogenes	0 0004	0 0007	0 0008	0 0008
A cloacae	0 0004	0 0007	0 0008	0 0008
E coli	0 0002	0 0005	0 0004	0 0007
E communior	0 0004	0 0007	0 0006	0 0008
E acidilactia	0 0004	0 0006	0 0006	0 0008
P aeruginosa	0 0002	*	0 0004	0 0005
P ovalis	0 0002	0 0005	0 0004	0 0006
P graveolens	0 0001	*	0 0002	0 0005
P syncyanea	0 0002	*	0 0004	0 0005
P mucedolens	0 0002	*	0 0004	0 0005
S marcescens	0 0006	0 0007	0 0008	0 0010
R agilis	0 0001	0 0005	0 0004	0 0006
R rosaceous	0 0001	0 0005	0 0004	0 0006
Torula rosea	0 0020	0 0011	†	0 0080
F sauveolens	0 0006	0 0007	0 0008	0 0008
A lipolyticum	0 0006	0 0007	0 0008	0 0010
B subtilis	0 0006	0 0008	0 0008	0 0010
B mesentericus	0 0004	0 0007	0 0006	0 0008
B mycoides	0 0004	0 0007	0 0006	0 0008
B fusiformis	0 0002	0 0005	0 0004	0 0097
B meliens	0 0004	0 0008	0 0006	0 0010
C intermedium	0 0004	0 0007	0 0006	0 0008
A faecalis	0 0004	0 0007	0 0006	0 0008
P vulgaris x 19	0 0004	0 0007	0 0006	0 0008
S aureus	0 0004	0 0007	0 0008	0 0008
S candidus	0 0002	0 0007	0 0006	0 0008
S albus	0 0002	0 0005	0 0006	0 0007
S flava	0 0002	0 0005	0 0004	0 0006
Sarcina lutea	0 0002	0 0005	0 0004	0 0007

* No growth in lowest concentration employed
† Growth in highest concentration employed

0 4 M depressed slightly or had no effect on the toxicity of cerium chloride and increased slightly or had no effect on the toxicity of cerium sulfate Sodium sulfate in 0 1 M concentration markedly reduced the toxicity of cerium sulfate (table 3), but was without significant effect when used with cerium chloride

Magnesium chloride (0 5 M) generally diminished the toxicity of cerium chloride, but magnesium sulfate (0 5 M) was without effect   The chlorides (0 5 M) of calcium and barium slightly increased the toxicity of cerium chloride, whereas barium sulfate, lithium chloride, ammonium sulfate, and ammonium chloride were without significant effect

The toxicity of cerium chloride for fungi   In all the preceding experiments it was observed that the *Torula* culture was far more tolerant of the cerium compounds than were the bacteria   In order to determine whether other common fungi are equally tolerant of cerium, a yeast extract glucose peptone medium was prepared with concentrations of cerium chloride sufficient to inhibit all the bacteria employed in this study   Thirty-five strains of fungi were inoculated on the media by streaking, and the results were read after incubation for 2 days at room temperature

The following organisms were employed   *Debaromyces tyrocola, Endomyces hordei, Monilia krusei, Mycoderma valida, Pichia farinosus, Saccharomyces cerevisiae* Frohberg, *Saccharomyces* of Curtis, *Saccharomyces cerevisiae* Saaz, *Schizosaccharomyces mellacei, Torula* "Hansen" sp , *Torula humicola, Torula mucilaginosa, Torula spherica, Torula datilla, Torula colliculosa, Torula sanguinea, Torula* "pink" sp , *Torula fructicola, Torula liconde, Torula fermentati, Torula kefyr, Torula lactosa, Torula candida, Zygosaccharomyces priorianus, Zygosaccharomyces chevalieri, Vermicularia* sp , *Fusarium* sp , *Phytophthora* sp , *Neocosmospora* sp , *Dothiorella* sp , *Cunninghamella* sp , *Trichoderma* sp , *Pythium* sp , *Rhizopus* sp , and *Aspergillus* sp

All the fungus cultures grew as well in the presence of 0 0014 M cerium chloride, the highest concentration employed, as in the control medium

The bacteriostatic activity of lanthanum and thallium   The toxicity of lanthanum chloride and thallium nitrate for the selected bacteria was determined by the same methods employed in the preceding experiments   The results are presented in table 4   The order of toxicity of lanthanum and thallium was found to be approximately the same as that of cerium   Again, some species were observed to be relatively more resistant than others   The organisms most tolerant of the salts were found to be certain species of *Bacillus, Serratia marcescens, Sarcina conjunctivae, Achromobacter lipolyticum,* and *Torula rosea*   The most susceptible organisms were species of the genus *Pseudomonas*

The salts of cerium, lanthanum, and thallium were found to be definitely more toxic for the bacteria than for the fungi included in this study

The 39 species of bacteria were prevented from growth in concentrations of cerium chloride varying from 0 0006 to 0 0012 M, in cerium nitrate from 0 0004 to 0 0008 M, in cerium sulfate from 0 0006 to 0 0008 M, in lanthanum chloride from 0 0002 to 0 0008 M, and in thallium nitrate from 0 0005 to 0 0010 M

The toxicity of cerium sulfate for most bacteria was reduced by the addition of sodium sulfate (0 05 M) to the medium, and the toxicity of cerium chloride was generally decreased by the addition of magnesium chloride (0 05 M)   The addition of other salts had little effect on the toxicity of cerium

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STUDIES ON THE QUANTITATIVE DIFFERENTIAL ANALYSIS OF MIXTURES OF SEVERAL ESSENTIALLY PURE PENICILLIN TYPES

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Since the work of Schmidt, Ward, and Coghill (1945) on a method for differentiating various types of penicillins by means of two assay organisms, very little further work has been published in this connection That various organisms respond to different penicillin types differently is well known (Veldee *et al*, 1945, Welch *et al*, 1944, Libby and Holmberg, 1945, Eagle, 1946, Eagle and Musselman, 1946, etc), but few specific attempts have been made to develop a quantitative test for specific penicillin types based on differential response of the organisms studied A relatively rough *in vivo* differential assay procedure was recently proposed by Buck, Farr, and Schnitzer (1946) in which *Borre'lia* infections in mice were used

With the recognition of the various penicillin types have come improved methods for separating mixtures of penicillins into the pure components (Fischbach *et al*, 1946, Craig *et al*, 1946) As these purification methods have been increasingly used, the need for accurate differential biological assay procedures has been felt more and more The recent publication of Higuchi and Peterson (1947) presents a procedure with which they attempted to fill this need

The method described by these authors employs three test organisms *Staphylococcus aureus* 209-P, *Bacillus brevis*, and "organism E " Using a turbidimetric test, they reported that it was possible by their method to estimate with fair accuracy the composition of mixtures consisting of penicillins G, K, and X They noted that the assay procedure was based on several assumptions, one of which was that "the effects of penicillins in mixtures on assay organisms are additive "

Since systematic studies on this latter question were under way in this laboratory with *Staphylococcus aureus* Heatley, the work was augmented after obtaining cultures of *B brevis* and "organism E" through the kindness of Dr Peterson The present paper includes studies on the effects of known mixtures of several penicillin types on these three species of organisms

A cursory survey of the literature leads one to believe that activities assigned to each of the various penicillin types actually have been established in some cases with mixtures of several types of penicillin For example, penicillin F, has been reported to have activities of 1,440 to 1,490 units per mg (Schmidt, Ward, and Coghill, 1945) and 1,550 units per mg (Higuchi and Peterson, 1947) Values of 845 to 935 units per mg (Schmidt *et al*, 1945), 850 (Coghill and Koch, 1945), 900 (Welch *et al*, 1944), and 1,000 units per mg (Libby and Holmberg,

1945) have been assigned to penicillin X   The slight shift in the assigned activity of penicillin G from 1,650 units per mg (Welch *et al*, 1944) to 1,667 units per mg (Veldee *et al*, 1945) with consequent slight changes in the relative activities of the other penicillin types cannot account for these discrepancies The various activities reported may be attributable to strains of organisms used for assay, to the assay procedures themselves, or to the varying degrees of pu rity of the preparations used

Since the chief purpose of the present work was to study the effect, if any, of one penicillin type on the action of another, efforts were made to use only peni cillin preparations the purity of which were as thoroughly established as pos sible

PENICILLIN PREPARATIONS USED

Penicillin G[1] (*Cra A-328-36*)   This is a crystalline sodium salt prepared from commercial penicillin by chromatography and recrystallized several times The chemical analysis agreed well with theoretical

$$\text{Found} \quad C, 53\ 81, H, 4\ 85$$
$$\text{Calc} \quad\ \ C, 53\ 92, H, 4\ 81$$

Craig countercurrent distribution studies of this preparation revealed that 90 per cent (by weight, 93 per cent of the activity) was contained in the main band, indicating it to be an essentially homogeneous material   The remaining 10 per cent consisted of inactive impurities, possibly inactivation products formed during the distribution experiment

Bioassays with *Staphylococcus aureus* Heatley against previously well estab lished standards showed its activity to agree very well with the defined activity of penicillin G, i e, 1,667 units per mg, suggesting a purity at least as good as any of the materials used in establishing such standards

Penicillin K[1] (*AV-73*)   This is a crystalline ammonium salt obtained by partition chromatography   The chemical analysis agreed well with the theo retical

$$\text{Found} \quad C, 53\ 34, 53\ 10, H, 8\ 05, 8\ 12, N, 11\ 62, S, 8\ 92, \text{Moisture, } (H\ 0)$$
$$\qquad\qquad 3\ 46$$
$$\text{Calc} \quad\ \ C, 53\ 46, H, 8\ 13, N, 11\ 69, S, 8\ 99$$

As will be shown later, careful bioassay of this preparation gave an activity of 2,540 units per mg, which is about 10 per cent higher than the figure of 2,300 usually assigned to penicillin K (Coghill and Koch, 1945)   The subtilis staph ylococcus ratio of 0 36 was in good agreement with that reported to be char acteristic of penicillin K (Coghill and Koch, 1945)

Penicillin X (*NRRL-1717-39A*)   The penicillin X used for these studies was supplied us through the kindness of Dr F H Stodola, of the Northern

Regional Research Laboratory, who described it as an analytically pure preparation having the following analysis

Calc for $C_{16}H_{17}N O_6S Na$ C, 51 6, H, 4 60
Found   C, 51 8, H, 4 89

Their bioassays with *Staphylococcus aureus* (strain not specified) indicated an activity of 920 units per mg

ASSAY PROCEDURE

Higuchi and Peterson (1947) in their differential assay procedure plotted turbidimetric readings of growth against units of penicillin per ml   When large numbers of assays are involved, a technique similar to that used for streptomycin assay (Donovick *et al*, 1945) has proved preferable in our hands   It is perhaps true that readings of partial inhibition, as were done by Higuchi and Peterson (1947), may be more accurate when the curves, obtained by plotting turbidimeter readings against units of penicillin per ml, are not steep, which was the case with their strain of *Staphylococcus aureus*   On the other hand, for organisms such as *B brevis* and "organism E," the curves were very steep, showing a change from little inhibition to almost complete inhibition over a very narrow range of penicillin concentrations   Hence, it appeared to us that little could be gained through the use of a turbidimeter for reading end points   The three test organisms used for the present work were *Staphylococcus aureus* Heatley and, as already indicated, two species used by Higuchi and Peterson (1947), viz , *B brevis* and "organism E "

Sixteen-hour cultures of the three organisms were diluted as follows *Staphylococcus aureus* Heatley, 1×10^{-6} in yeast beef broth (Difco), *B brevis*,[2] $0 25 \times 10^{-5}$ in "Peterson B"[3] broth, and "organism E", 1×10^{-5} in "Peterson E"[3] broth   These dilutions gave counts of approximately 1,000 organisms per ml

Two-ml volumes of inoculated broths were dispensed with sterile automatic syringes into sterile tubes measuring 13 by 100 mm   The penicillin solution to be assayed, appropriately diluted, was then added to the 2-ml volumes of inoculated broth by means of acid-cleaned, sterile, 0 2-ml Kahn pipettes in the following amounts 0 10, 0 088, 0 077, 0 068, 0 059, 0 052, 0 046, 0 040, 0 035,

[2] It was found to be advisable to grow *B brevis* in a shallow layer of broth to obtain sufficiently heavy growth in 16 hours to allow the indicated dilution for the tests
[3] These media were used by Dr Peterson in some of his early work and were recommended to us by him (personal communication)   They had the following compositions

	Peterson 'B' broth g/liter	Peterson ' E ' broth g/liter
Peptone	6 0	6 0
Yeast extract (Difco)	3 0	3 0
Glucose	1 0	2 0
K HPO$_4$	3 2	0 5
KH$_2$PO$_4$	2 0	5 0
pH	6 8	6 0

and 0 030 ml   The racks containing the tubes of inoculated broth were kept at 5 C prior to the addition of penicillin   Three racks at a time (i e , one rack of each of the three test organisms) were removed from the icebox, the penicillin was added, and the racks were returned at once to the cold room (5 C) When penicillin had been added to all the racks for a given day, they were all placed in the appropriate incubators[4] at one time and incubated for 15½ to 16½ hours

The tests, after the tubes were vigorously shaken, were read under a fluorescent day lamp   Absence of growth was recorded as (−), an intermediate degree of growth as (±), and almost complete or complete growth as (+)   The end point was considered to be the last (−) in a (−) (+) series, and the mid point between (−) and (±) in a (−) (±) (+) series   Since in the present investigations the concentrations (by weight) of penicillin in the solutions tested were known, the minimal inhibiting concentrations (M I C) were readily calculated from the volume of penicillin solution added to the end point tube

Early in the present studies aqueous solutions of each type of penicillin were prepared from carefully weighed samples   The desired mixtures were made by mixing appropriate proportions of the various solutions   All samples were then dispensed in acid-cleaned, sterile ampoules, in ca 1-ml amounts, and the ampoules sealed and frozen in a CO_2-alcohol bath   The ampoules were then stored in a CO_2 box until used   When assays were to be made, enough ampoules for that days' work were thawed, and the contents were diluted with distilled water and assayed

It will be noted that Higuchi and Peterson (1947) expressed penicillin concentrations in terms of the standard unit, "in order to compare the results obtained   with previous results "   As a consequence, the algebraic expressions which they derived for calculating compositions of penicillin mixtures yielded results in units per cent   The present authors feel that where three test organisms and three or more types of penicillin are involved the use of units leads to confusion and obscures various relationships   This will be discussed more fully below, but suffice it to say for the moment that all M I C data were gathered and are here reported in terms of actual weights of penicillin per unit volume, and the equations given below yield results in percentage by weight   It is obvious that the composition of a mixture containing, e g , v per cent G and 50 per cent K by weight is quite different from one the active of which consists of 50 units of G and 50 units of K

Tests on known mixtures of two or more types of penicillin were always accompanied by controls consisting of tests on solutions containing separate the individual components involved in the mixtures   Hence, large number assays of the solutions containing only single types of penicillin were conducted In table 1 are shown the results of the tests on these control solutions

Comparison of the M I C values shown in table 1 with those given by Higuchi and Peterson (1947) reveals surprising differences in findings   The these differences is uncertain, but several explanations suggest them

[4] *Staphylococcus aureus* and *B brevis* were incubated at 37 C, "organism D" at

TABLE 1

Minimal inhibiting concentrations of penicillin in terms of weight

PENICILLIN	EXPERI MENT NO	MIC		
		S aureus (Heatley)	B brevis	"Organism E"
		µg/ml	µg/ml	µg/ml
G (CrA 328-36)	1	0 00790 (27)*	0 0125 (25)	0 0311 (23)
G (CrA-328 36)	2	0 00770 (126)	0 0150 (120)	0 0308 (120)
G (CrA-328-36)	3	0 00720 (65)	0 0145 (60)	0 0329 (55)
Average G		0 00755 ± 0 735%†	0 0146 ± 0 957%	0 0317 ± 0 897%
K (AV-73)	1	0 00463 (8)	0 0458 (8)	0 0686 (8)
K (AV-73)	2	0 00515 (31)	0 0475 (28)	0 0690 (29)
K (AV-73)	3	0 00480 (24)	0 0458 (26)	0 0641 (25)
Average K		0 00495 ± 1 29%	0 0465 ± 1 17%	0 0670 ± 2 14%
X (NRRL 1717 39A)	1	0 0136 (51)	0 0535 (49)	0 0283 (43)
X (NRRL-1717 39A)	2	0 0133 (26)	0 0535 (22)	0 0277 (24)
X (NRRL 1717-39A)	3	0 0143 (24)	0 0585 (24)	0 0289 (24)
X (NRRL-1717-39A)	4	0 0154 (30)	0 0550 (29)	0 0342 (31)
Average X		0 0140 ± 1 04%	0 0545 ± 1 10%	0 0295 ± 1 49%

* Figure in parenthesis represents number of assays conducted on the specific sample
† The standard errors shown were calculated on the results of the total number of assays carried out with a specific preparation   The authors are indebted to Mr Ross Blue of E R Squibb & Sons for this statistical analysis

It is possible that slight differences in media, or perhaps in variations occurring in the cultures, between the time Higuchi and Peterson conducted their tests and the time we received these cultures may have accounted in part for these differences   Probably even more important was that in the present work complete inhibition was taken as the end point, whereas some point of partial inhibition (but which is not clearly indicated) was used as the end point by Higuchi and Peterson (1947)

The latter authors reported M I C values, for their strain of *Staphylococcus aureus* (209-P), which are close to twice as great as those found for *Staphylococcus aureus* Heatley in the present work   Though the strains of *B brevis* and "organism E" used for these studies were the same as those used by the foregoing authors, the minimal inhibiting concentrations reported by them for the various penicillin types studied are quite different from those reported here It is interesting, therefore, to note how the results compare when the present data are converted into units

Assigning to penicillin G its defined activity of 1,667 units per mg, the K penicillin used in the present work would have an activity of $1{,}667 \times \frac{7\,55}{4\,95} = 2{,}540$ units per mg, and the X used would have $1{,}667 \times \frac{7\,55}{14\,0} = 898$ units per

TABLE 2

M nimal inhibiting concentrations of penicillin in terms of units

TEST ORGANISM	PENICILLIN TYPE	ACTIVITY OF PENICILLIN UNITS PER MG	MIC		RATIO OF MIC S IN TERMS OF UNITS	
			µg per liter	Units per liter	G/K	G/A
S aureus (Heatley)	G	1,667	7 55	12 6		
S aureus (Heatley)	K	2,540	4 95	12 6	1	
S aureus (Heatley)	X	898	14 0	12 6		1
B brevis	G	1,667	14 6	24 3		
B brevis	K	2,540	46 5	118 1	0 206	
B brevis	X	898	54 5	48 9		0 49
"Organism E"	G	1,667	31 7	52 8		
"Organism E"	K	2,540	67 0	170 1	0 310	
"Organism E"	X	898	20 5	26 5		1 97

mg   On the basis of these potencies, conversion of the M I C values shown (in terms of weight) in table 1 to M I C in terms of units give the results shown in table 2

Thus, despite the differences between the absolute (weight) M I C values reported here and by Higuchi and Peterson (1947), equations very similar to those of the latter authors, based on relative (unitage) M I C ratios, may be set up

STUDIES WITH KNOWN MIXTURES CONTAINING TWO TYPES OF PENICILLIN

To determine whether the effects of the various types of penicillins were truly additive, mixtures containing two types were first studied   The findings with such two component mixtures are shown in tables 3, 4, and 5   Studies were then undertaken with three component mixtures and these results are listed in table 6   The values listed under the columns headed "theor" were calculated by means of equations (1), (2), and (3) below after substituting in the known values of a, b, and c   These equations hold true only in so far as the effects of the penicillins are additive, in which case there would be a direct proportionality between the composition of a given mixture and the M I C values of this mixture for the three test organisms

The following equations relate the concentration (in terms of weight per volume) of mixed penicillins at the end point to the composition of the mixture

In a mixture of penicillins, let—

a = per cent, by weight, of penicillin G

b = per cent, by weight, of penicillin K

c = per cent, by weight, of penicillin X

MsG = M I C of pure penicillin G for *Staphylococcus aureus*

MsK = M I C of pure penicillin K for *Staphylococcus aureus*

MsX = M I C of pure penicillin X for *Staphylococcus aureus*

Similarly let—

MbG = M I C of pure penicillin G for *Bacillus brevis*

MeG = M I C of pure penicillin G for "organism E," etc

Ms = M I C of mixture of penicillin for *Staphylococcus aureus*

Mb =M I C of mixture of penicillin for *Bacillus brevis*, etc

TABLE 3

Minimal inhibiting concentrations of known mixtures of penicillins G and K

COMPOSITION* OF MIXTURE		S AUREUS			B BREVIS			'ORGANISM E"		
		Ms μg per liter			Mb μg per liter			Me μg per liter		
Per cent G	Per cent K	Theor	Found	Found/Theor	Theor	Found	Found/Theor	Theor	Found	Found/Theor
100	0	7 55†	7 55	1 00	14 6†	14 6	1 00	31 7†	31 7	1 00
90	10	7 16	7 50	1 05	15 7	15 5	0 99	33 4	35 5	1 06
80	20	6 83	7 00	1 02	16 9	17 5	1 03	35 4	37 5	1 06
65	35	6 38	7 18	*1 12*	19 2	20 0	1 04	38 9	44 3	*1 14*
50	50	5 98	7 01	*1 17*	22 2	22 0	0 99	43 1	49 6	*1 15*
35	65	5 63	6 75	*1 20*	26 4	28 5	1 08	48 3	54 6	*1 13*
20	80	5 31	6 34	*1 19*	32 4	33 5	1 03	54 7	55 0	1 00
10	90	5 12	5 50	1 07	38 2	39 0	1 02	60 4	65 0	1 08
0	100	4 95†	4 95	1 00	46 5†	46 5	1 00	67 0†	67 0	1 00

Figures in italics indicate that the data from which the ratios were derived were analyzed statistically and that the deviations from 1 00 were significant

* Composition in terms of grams of given penicillin per 100 grams of total penicillin

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point

TABLE 4

Minimal inhibiting concentrations of known mixtures of penicillins G and X

COMPOSITION* OF MIXTURE		S AUREUS			B BREVIS			'ORGANISM E"		
		Ms μg per liter			Mb μg per liter			Me μg per liter		
Per cent G	Per cent X	Theor	Found	Found/Theor	Theor	Found	Found/Theor	Theor	Found	Found/Theor
100	0	7 55†	7 55	1 00	14 6†	14 6	1 00	31 7†	31 7	1 00
90	10	7 92	8 82	*1 11*	15 8	17 0	1 08	31 4	35 4	*1 18*
80	20	8 32	8 87	*1 06*	17 1	17 5	1 02	31 2	34 0	*1 09*
65	35	9 00	9 00	1 00	19 6	20 5	1 02	30 8	33 5	1 09
50	50	9 84	10 5	1 07	23 0	22 5	0 98	30 5	32 5	1 06
35	65	10 8	11 5	*1 07*	28 8	27 0	0 94	30 2	31 6	*1 04*
20	80	12 0	13 3	*1 11*	35 2	36 0	1 02	29 9	31 9	*1 06*
10	90	12 9	13 9	*1 08*	42 7	43 0	1 01	29 7	32 5	*1 09*
0	100	14 0†	14 0	1 00	54 5†	54 5	1 00	29 5†	29 5	1 00

Figures in italics indicate that the data from which the ratios were derived were analyzed statistically and that the deviations from 1 00 were significant

* Composition in terms of grams of given penicillin per 100 grams of total penicillin

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point

is apparently not statistically significant, yet the tendency appears to be in the same direction

When the mixture consisted of penicillins G and X, deviations reached maxima in two regions, one in the vicinity of 10 per cent X and another at 80 to 90 per cent X for both *Staphylococcus aureus* Heatley and "organism E " The picture appeared to be similar here in the case of *B brevis*, but again the deviations from theory were not statistically significant

The data on staphylococcus and "organism E" in K-X mixtures were very inconclusive except in the vicinity of 50-50 mixtures in which the amount of penicillin required to inhibit was again significantly greater than expected In this case the data on *B brevis* were quite clear-cut   Significantly more penicillin was required to inhibit this organism than would be expected in mixtures covering the range of 50 to 80 per cent X

It is of interest to note that in none of the cases studied was the experimental M I C significantly less than the theoretical figure   It would appear, therefore, that in two component mixtures of penicillins, one penicillin may interfere with the action of the other, thereby requiring a greater total amount of penicillin to cause inhibition than might be expected   Since very little is understood of the mode of action of the penicillins, it is not possible at present to explain this apparent interference   It is not even clear whether these compounds act within the bacterial cell or upon the cell surfaces, nor, in fact, whether all the penicillins inhibit growth in identically the same fashion

If, as a working hypothesis, one were to assume that the penicillins act within the cell rather than upon the surface, then one might tentatively propose that the apparent interference may actually be caused by differential adsorption of the various penicillins at the cell surface as well as differential diffusion into the cell   This would result in the composition of the penicillin mixture inside the cell being different from that outside   For example, it can be seen in table 3 that for *Staphylococcus aureus* Heatley the experimental M I C of a mixture containing 20 per cent G and 80 per cent K is equal to the theoretical M I C of a 65 per cent G and 35 per cent K mixture   On the other hand, for " organism E" the experimental M I C of a mixture containing 35 per cent G and 6 per cent K is equal to the theoretical M I C of a 20 per cent G and 80 per cent K mixture   If differential adsorption is in fact the reason for the observed interference, then one might expect penicillin K to be adsorbed more readily than penicillin G by *Staphylococcus aureus* Heatley and the reverse to be true for "organism E " Studies on adsorption of penicillin by bacteria which under way in this laboratory (Rake *et al* , to be published) may perhaps weight for or against such a hypothesis

Of course, the interference may be due to competition at a site of action penicillin within the bacterial cell, but little can be said about this in the present state of knowledge of the mode of action of the penicillins

STUDIES WITH MIXTURES CONTAINING THREE TYPES OF PENICILLIN

The question of deviation of behavior from the expected becomes more difficult to answer with the increase in the number of penicillins involved

was assumed, for purposes of calculation, that the effects of the penicillins were additive, and equations were derived expressing the relationship between the M I C of a mixture and its composition by weight—equations (1), (2), (3), and (4) Using these equations the "theoretical" values of Ms, Mb, and Me for 10 three-component mixtures were calculated, and at the same time these values were determined experimentally for these mixtures The comparison of these two sets of data is shown in table 6

It will be noted that with *Staphylococcus aureus* and *B brevis* the ratios between "theoretical" and experimental M I C values were usually very close to 1 0, whereas with "organism E" in 5 out of 10 mixtures the ratios indicated

TABLE 6

Minimal inhibiting concentrations of known mixtures of penicillins G, K, and X

COMPOSITION* OF MIXTURE			S AUREUS			B BREVIS			"ORGANISM E"		
			Ms µg per liter			Mb µg per liter			Me µg per liter		
Per cent G	Per cent K	Per cent X	Theor	Found	$\frac{Found}{Theor}$	Theor	Found	$\frac{Found}{Theor}$	Theor	Found	$\frac{Found}{Theor}$
100	0	0	7 55†	7 55	1 00	14 6†	14 6	1 00	31 7†	31 7	1 00
0	100	0	4 95	4 95	1 00	46 5	46 5	1 00	67 0	67 0	1 00
0	0	100	14 0	14 0	1 00	54 5	54 5	1 00	29 5	29 5	1 00
80	10	10	7 50	7 50	1 00	17 0	16 5	0 97	33 2	38 0	1 14
60	20	20	7 45	7 50	1 01	20 4	19 5	0 96	34 9	36 5	1 05
40	40	20	6 75	6 50	0 96	25 2	25 0	0 99	39 5	39 5	1 00
40	20	40	8 19	8 50	1 04	25 6	25 5	1 00	34 4	38 0	1 10
33 3	33 3	33 3	7 39	7 50	1 01	27 7	25 5	0 92	37 3	39 0	1 05
20	60	20	6 17	6 50	1 05	33 0	35 5	1 07	45 5	48 0	1 05
20	40	40	7 36	8 00	1 09	33 8	37 0	1 09	38 8	44 0	1 13
20	20	60	9 11	9 40	1 03	34 5	33 5	0 97	33 7	38 0	1 13
10	10	80	11 0	11 5	1 04	42 2	43 4	1 03	31 5	37 0	1 17
10	80	10	5 49	4 95	0 90	38 6	37 5	0 97	54 0	55 0	1 02

* Composition in terms of grams of given penicillin per 100 grams total penicillin
† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point

that from 10 to 17 per cent more penicillin than expected was required Although the experimental error in this work was probably no greater than that with the two component mixtures (since the two sets of data were gathered under identical conditions), the error in the "theoretical" figures would be statistically higher, having been derived algebraically from data on each of the three types of penicillin used, and the total error would contain errors from figures on each type of penicillin Consequently, no attempts were made to establish the degree of significance of these deviations Instead, attention was turned to the question of how well the compositions of the various mixtures could be calculated from the experimental data

Going back to equations (1), (2), (3), and (4) it can be seen that, by solution with simultaneous equations, the concentration (per cent by weight of total

such precision cannot be expected   Hence, it is of interest to know how changes
in the M I C values will affect the location of the point or points of intersection
To demonstrate this effect each of the three M I C values used in plotting the
lines of figure 2 were arbitrarily increased by 10 per cent and new lines plotted
corresponding to these new M I C values   This gave the dotted lines shown in
figure 2

It is at once evident that a 10 per cent error in both Ms and Mb does not
shift the point of intersection of these lines nearly so much as it shifts the point
of intersection of the Ms and Me lines   In fact, an error of 10 per cent in the
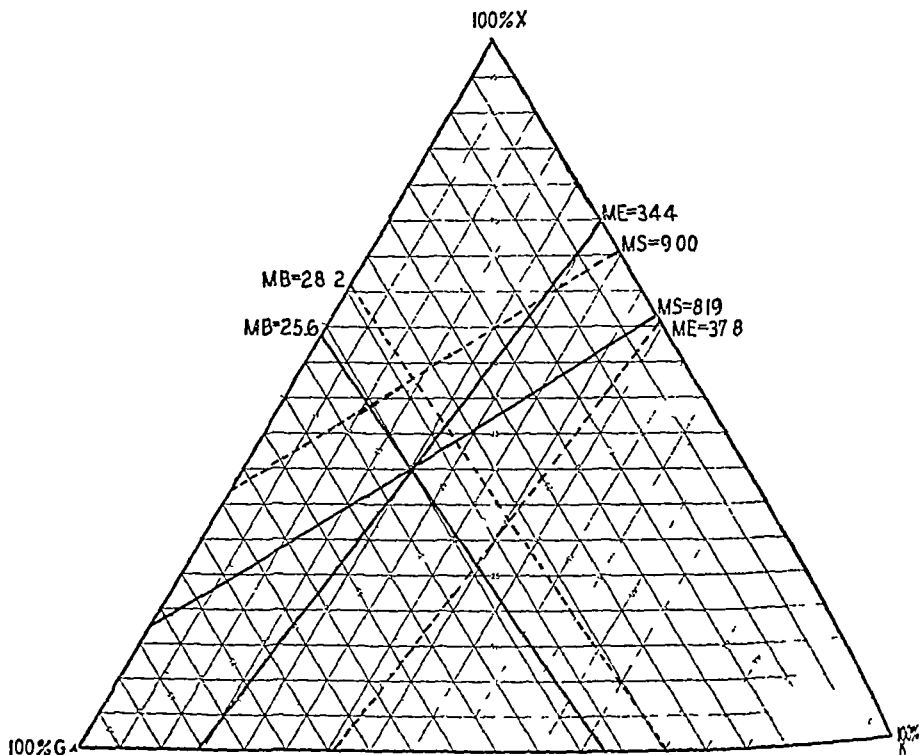
FIG 2 THE EFFECT OF EXPERIMENTAL ERRORS IN M I C VALUES ON
CALCULATED COMPOSITION OF PENICILLIN MIXTURES

Me value causes its line to intersect the Ms line somewhere off the graph (at
an imaginary point, since in the case demonstrated this would mean a negative
value for a, or less than 0 per cent penicillin G)   Examination of this graph
indicates that *Staphylococcus aureus* and "organism E" make a poor pair fo
quantitative differential analysis of penicillin mixtures   *Bacillus brevis* and
"organism E" are a somewhat better pair, but the best pair here tested is *Sta
phylococcus aureus* and *B brevis*   The graph also indicates why the use of the
experimentally determined Ms and Me values (table 6) when used in equations
(8), (9), and (10) gave such meaningless values for a', b', and c' (table 7) It

can also be seen why the use of the experimentally determined Ms and Mb values gave fairly good figures for a, b, and c (table 7) when equations (5), (6), and (7) were employed Thus the use of triangular co-ordinate graph paper in this manner could be of great aid in the search for organisms best suited for quantitative differential analysis of penicillin mixtures

The 10 three-component mixtures which were studied were analyzed graphically in this manner, calculating the composition from the experimentally determined Ms and Mb values In figure 3 the compositions calculated in this
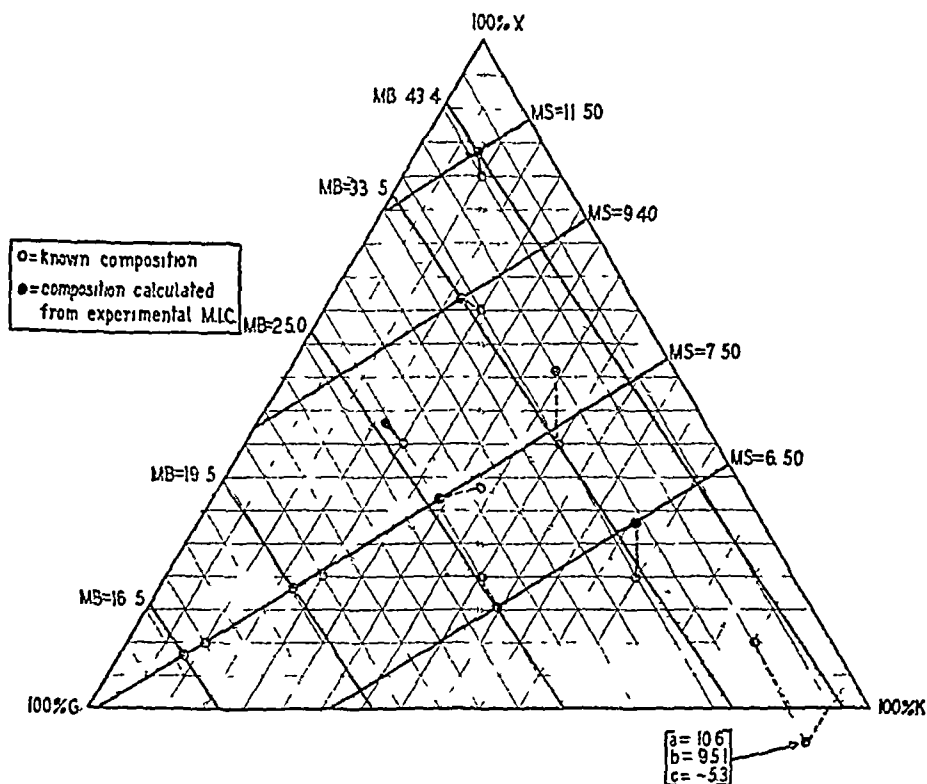
Fig 3 Differential Analysis of Penicillin Mixtures Using Staphylococcus aureus and Bacillus brevis

manner are shown as solid points, and the known composition of the mixtures are shown as open circles To simplify the appearance of this graph for purposes of photography some of the Ms and Mb lines have been omitted, but their points of intersection (solid dots) are shown

It will be noted that only when the content of penicillin G fell below ca 20 per cent of the total penicillin present in a mixture were there marked differences between the estimated and known compositions Even these differences may have been within experimental error, except for the case of the mixture consisting of 10 per cent G, 80 per cent K, and 10 per cent X In this case the Ms value was equal to that for pure K This resulted in the point, representing

such precision cannot be expected   Hence, it is of interest to know how change
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To demonstrate this effect each of the three M I C values used in plotting th
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FIG 2 THE EFFECT OF EXPERIMENTAL ERRORS IN
CALCULATED COMPOSITION OF PENICILI 1

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experimentally determined Ms
(8), (9), and (10) gave such ·

the staphylococcus brevis "organism E" ratio of penicillin F is in the vicinity of $8\,4\ 32\ 58 = 1\,3\,8\,6\,9$

In the final purification of preparations of the various penicillin types these triple ratios, as has become the custom to call them, have been considerable and as guidelines

SUMMARY

Using *Staphylococcus aureus* Heatley, *Bacillus brevis*, and Peterson's "organism E" as test organisms, it has been shown that in mixtures of two types of penicillin, more penicillin is required to cause inhibition of growth than would be expected from data on the actions of the individual penicillin types   Until the mode (or modes) of action of the penicillins are better understood, this interference on the part of one type of penicillin with the action of another cannot be explained   However, it is tentatively proposed that this phenomenon may be caused by differential adsorption of the various penicillins at the cell surface as well as differential diffusion into the cell

Equations are given which show the algebraic relationship between the composition of a given penicillin mixture and the weight of total mixed penicillin required to inhibit growth   Through the use of these equations, as well as through the use of a graphic procedure employing triangular co-ordinate paper, it has been shown that only two test organisms are needed for the analysis of mixtures containing three types of penicillin and that *Staphylococcus aureus* Heatley and "organism E" make a poor pair of organisms for such quantitative differential analyses   A better pair of test organisms is that of *Staphylococcus aureus* Heatley and *B brevis*   However, evidence is also presented to show that even with this pair of organisms relatively slight variations in the experimentally determined minimal inhibiting concentrations cause significant variations in the calculated composition of such mixtures   Hence, such procedures at best give only rough approximations of the composition of penicillin mixtures and are most valuable in the final purification steps of single penicillin types

The graphic procedure described may prove to be of assistance in finding the best test organisms for such differential analyses

It has been pointed out that when essentially pure penicillins are involved there are advantages in calculating minimal inhibiting concentrations in terms of weight instead of in units

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A BACTERIAL SPRAY APPARATUS USEFUL IN SEARCHING FOR ANTIBIOTIC-PRODUCING MICROORGANISMS

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A number of writers have reviewed the various methods available for isolating antibiotic-producing microorganisms (Waksman, 1945, Pinschmidt and Levy, 1944) These methods have different disadvantages Some involve individual testing of every organism obtained and are, therefore, extremely laborious Others, as the bacterial agar plate, appear to be of questionable value (Waksman and Schatz, 1946) Still others, for example, the crowded plate method (Stokes and Woodward, 1942), are objectionable because the less dominant soil organisms tend to be neglected and, in addition, the activity of the observed antibiotic colonies is not directed against a particular test organism but against some chance soil form which happens to be in the vicinity

The present communication describes a spray apparatus by means of which agar plates containing several or numerous soil colonies, for instance, may be conveniently inoculated with a desired test organism and then reincubated to detect the antibiotic-producing colonies The advantages of this treatment are apparent It permits immediate recognition of growth-inhibiting substances specifically directed against the test organism and emanating from antibiotic colonies on plates with numerous inactive organisms In this way individual testing of vast numbers of organisms present in the heterogeneous soil population, which would ultimately prove to be inactive, is obviated The many disadvantages attending flooding plates instead of spraying, such as smearing, spreading, and overgrowth of soil colonies which may render recognition and isolation of antibiotic-producing organisms impossible, are largely avoided The spraying technique has led with a minimum of labor to the isolation of a number of antibiotic-producing organisms including *Bacillus polymyxa*, which produces the antibiotic substance polymyxin (Stansly, Shepherd, and White, in press)

Although the spray apparatus has been designed for the specific purpose given, its application in other problems involving the seeding of agar plates suggests itself

THE SPRAY APPARATUS

Although a more elaborate spray apparatus has been constructed for the purpose described, a simple device which has served usefully for several years in this laboratory is shown in figure 1 This apparatus is constructed of readily available materials, requires no special skill in constructing, is simple to operate, and may be sterilized by autoclaving

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As shown in figure 1, the apparatus consists of a de Vilbiss medicinal atomizer (no 154) connected by friction to a compressed-air cut-off assembly (no 633) which has a convenient trigger control   The assembly is connected to the compressed-air line through a reducing valve and gauge

The nozzle is inserted into the lower end of the spray chamber through a small rubber stopper and glass tubing which is then pushed through a no 14 rubber stopper   A second glass tubing in the large stopper is connected to a water aspirator after first passing through a solution of phenol or other disinfectant

The spray chamber consists of a pyrex tube 9½ inches long, having an outside diameter of 3½ inches   At the upper end of the spray chamber where the agar plate is held, a gasket was constructed of ½-inch electrical tape and next to it a
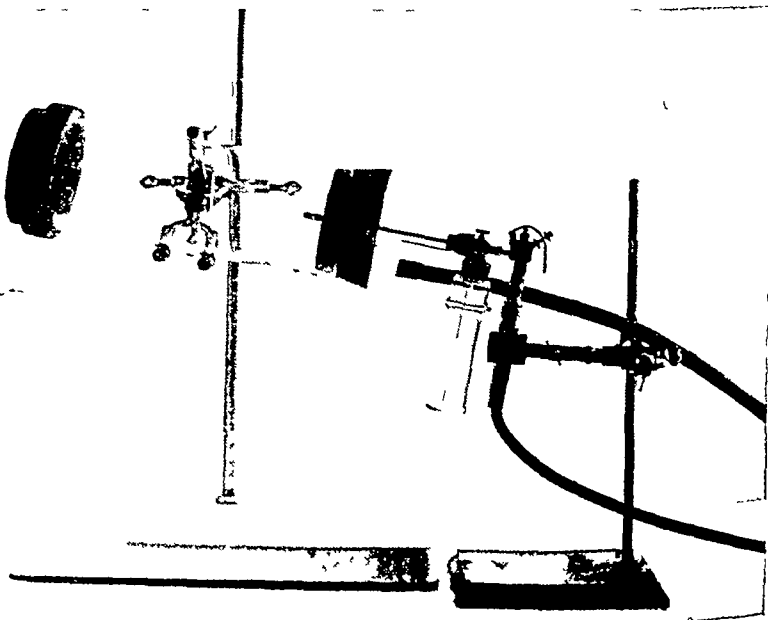
FIG 1 BACTERIAL SPRAY APPARATUS

barrier was built up of rubber tape covered by electrical tape   The gasket permits a reasonably tight fit of petri plates of normally varying diameter   The barrier allows the petri plates to be held firmly during spraying without danger of crushing the agar   In addition, it aids in making an efficient seal to prevent leakage of spray   The gasket and barrier have stood up extremely well upon repeated sterilization   Soft rubber might well replace the electrical and rubber tape used, but was not available at the time the apparatus was constructed

OPERATION

The spray bottle is filled with the bacterial suspension and screwed into the atomizer   The air is turned on to give a pressure of 5 to 10 pounds and the aspirator to give moderate suction, which is maintained until all the plates have

been sprayed   Aspiration serves a dual purpose   The reverse current produced prevents a hovering cloud of spray from leaking out between the removal of a sprayed plate and its replacement with a fresh one   A second function of aspiration is to dry the sprayed plates rapidly before removal

As a rule, a few brief sprays with several turns of the petri dish suffice to insure an even growth of the test organism over the whole surface of the petri dish Generally an undiluted or a tenth-diluted 24-hour broth culture of an organism such as *Salmonella schottmuelleri* is used   *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium* no 607 (A T C C), and *Mycobacterium ranae* have also given satisfactory results

Despite the somewhat crude gasket and barrier described, there is notable freedom from spray leakage judging from occasional checks made by exposing open agar plates in the vicinity of the target petri dish during spraying   There is likewise little or no leakage in the interval in which a sprayed plate is removed (after a minute of drying) and replaced with a fresh one, provided suction is maintained   If a negative pressure is not maintained, innumerable colonies will appear on the fresh plate   Naturally, good fit of all parts and adequate suction are important factors in minimizing spray leakage   Although the apparatus described is felt to be reasonably efficient and safe from this standpoint, it is not to be recommended for use with virulent pathogens capable of causing serious disease by ingestion or inhalation

Under optimum conditions, one plate per minute can be conveniently sprayed The apparatus may be disengaged at the junction of the atomizer and cut-off assembly   Without further manipulation the spray chamber, atomizer, bottle, suction hose, and disinfectant wash bottle may be placed in a pan and autoclaved   We do not feel it necessary to sterilize the cut-off assembly

SUMMARY

A method of searching for antibiotic-producing microorganisms involving the use of a bacterial spray apparatus is described   Details of the construction and operation of a simple spray device are given

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A SIMPLE METHOD FOR CONTROLLED EXPERIMENTATION ON THE PASSAGE OF MICROORGANISMS THROUGH THE DIGESTIVE TRACT OF INSECTS

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This paper is a preliminary report on a relatively new technique being employed for controlling the feeding of insects and the collection of their stools for microbiological studies [1] We are primarily interested in the possible role played by insects in the transmission of disease, particularly enteric diseases, through the agency of food which they may contaminate To the best of our knowledge no conclusive experiments have been attempted on completely controlled feeding of insects to study their ability to ingest, retain, and to disseminate specific microorganisms Papers by Steinhaus (1940, 1941) and Gier (1947) suggest a fertile field for investigations of this type

The latest complete survey on the relationship between microorganisms and insects is probably that presented by Steinhaus (1946), and even this excellent review offers little exact information on this particular phase of the subject Consequently, our experiments are based upon techniques perfected largely through trial and error We are indebted, however, to Dr Lawrence R Penner of the Zoology Department of the University of Connecticut for his valuable suggestions on the mounting and feeding of the live insects He has employed similar methods with flies in his studies on poliomyelitis virus

MATERIALS AND METHODS

Our experiments are being conducted with *Blaberus cranifer*, a large roach common in Florida Specimens are shipped to us by an express from Key West Because of its large size (about 3 to 5 cm long and 2 to 3 cm wide in the adult stage), this roach is particularly well adapted to our feeding studies in which not only the quality but also the quantity of food and organisms ingested can readily be controlled

By mounting these insects on blocks of paraffin (melting point 51 to 52 C) we have been able to keep them alive in battery jars under completely controlled conditions for weeks at a time The blocks are first softened by placing them in hot water and then are molded to fit the general contour of the back of the roach By momentarily flaming the paraffin it can be made to adhere when pressed to the back of an insect which has previously been placed in the freezing compartment of a refrigerator for about five minutes This chilling procedure tends to keep the roach relatively inactive during the mounting operation Once contact of the wings is established with the paraffin block, a firmer mount is prepared by embedding the edge of the wings with melted paraffin directed

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[1] Frings (1946) has discussed the history of the use of similar techniques by various authors for a number of purposes

to the area with an eye dropper   The insects are then placed on their back
and the blocks are attached to glass rods for mounting as is shown in figure 1
    Our early attempts to collect stool specimens met with failure when we tried
to suspend the insects in an upright position   Too often the roaches regurgitated
their food, and cultures made from the stools were contaminated with this re
gurgitated material   By mounting the subjects on their backs and by trimming
the wings at the posterior end it was possible to collect the stools in a satisfactory

FIG 1   THE METHOD OF FEEDING THE INSECT

manner   The comfort of the roaches when lying on their backs appears to be
optimum under the conditions of the test
    Stool specimens were collected directly on agar plates or on differential media
placed below the insect, and these media kept the stools from drying out   A
high humidity, one of the apparent prerequisites for keeping these roaches alive
for any length of time, was maintained by placing a small amount of water in the
bottom of the battery jar (figure 2)   By covering the top of the jar with multiple
layers of cheesecloth, contamination of the agar plates was minimized while
access of air was unimpaired

Hand feeding of sterile molar solutions of sucrose containing a trace of Difco yeast extract was accomplished with a tuberculin syringe fitted with a 20-gauge needle Any pure cultures of organisms to be fed were merely added to this sterile basic diet By forcing a drop of the liquid at a time from the needle and holding it near the mouth parts of the roaches, the insects soon learned to take the solution rapidly without any loss through spilling Dr Penner reports that he has kept flies alive for several months on nothing more than a |molar
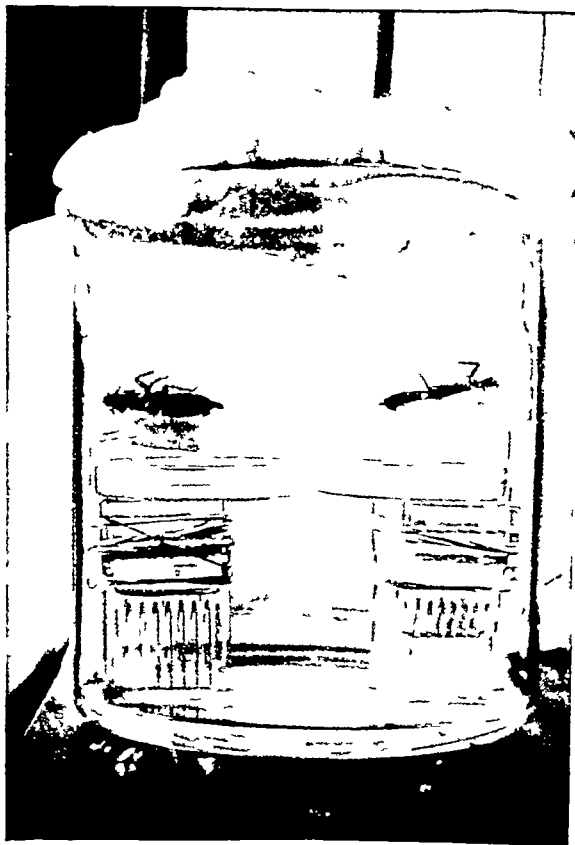
FIG 2 THE METHOD OF STORING THE MOUNTED INSECTS FOR EXPERIMENTAL WORK

sucrose solution fed in this manner The fluid intake varied considerably between insects, but in general each roach would consume an average of between 0 2 and 0 3 ml per day, all in a single morning feeding The quantity of fluid ingested tended to decrease the longer the roach remained mounted Trying to overfeed them always resulted in prompt regurgitation, an undesirable reaction which we soon learned to control

There is some evidence that storage of these roaches at about 30 C may keep them more active with a resulting increase in food intake This is desirable if frequent stools are to be passed Experiments are also in progress in which

we are attempting to feed more solids in the hope that we may be able to increase
the number and the quantity of stools passed

Stools were transferred from the moist agar surface and were emulsified in
several drops of nutrient broth   Streak plates were then made from the emulsion
on differential media, and isolations were fished for pure culture identification
Normal flora studies revealed that the species of aerobic organisms in the normal
stools of this particular species of roach are few in number, a factor which sim
plified our later studies with pure cultures of organisms fed to the subject
Further confirmation of normal stool flora for these insects is in progress together
with controlled feedings of cultures   The results of these investigations will
be reported in a future paper

ACKNOWLEDGMENT

SUMMARY

A relatively simple technique for controlled feeding of insects being employed
in microbiological studies is presented   Experiments designed to study both the
normal and the induced flora of stools from such insects can be materially ad
vanced by the elimination of ordinary body contaminants   A subsequent paper
will outline the results of studies now in progress on attempts to pass specific
organisms through the alimentary tract of *Blaberus cranifer*, a large roach com
mon in Florida   Similar techniques may be applied with flies and other insect
to see whether they are capable of serving as carriers of specific organisms over
a period of time

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AN ACTINOPHAGE FOR STREPTOMYCES GRISEUS[1] [2]

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Insufficient differentiation is frequently made between the production by a microorganism of an autolytic principle, or an agent which dissolves the cell of the organism producing it, and a phage or transferable principle, which is capable of dissolving not only the cells of the culture producing it but also those of other cultures of the same or other organisms upon transfer   This has often led to confusion in the interpretation of certain stages in the life cycle of the organism or of some of its metabolic processes   Although much light has been thrown in recent years on the nature and mode of action of phages of bacteria, the production of such agents by other microorganisms, notably fungi and actinomycetes, is still insufficiently understood   The significance of the ability of a phage or a viruslike agent to attack filamentous microorganisms in the practical utilization of such organisms for the production of various chemical agents has recently been emphasized in the discovery of a phage which has the capacity to attack streptomycin-producing strains of *Streptomyces griseus*

The term "bacteriophage" is usually applied to the virus or phage of bacteria, and the term "mycophage" to that of fungi   By analogy, the term "actinophage" may be used to designate the phage of actinomycetes   The origin of the phage, whether it is carried in the culture or brought in from outside like any other contamination, its nature and activity, and its similarity to bacterial phages form some of the most important problems in the elucidation of this natural phenomenon

HISTORICAL

The first recorded observations of the lysis of an actinomyces culture and of the significance of this reaction in the life cycle of the organism and in the production of new strains were made by Dmitrieff (1934) and by Dmitrieff and Soutieff (1936)   A culture of an organism called by the authors *Actinomyces bovis*, and evidently belonging, according to modern concepts, to the genus *Streptomyces*, was found to undergo lysis in various media   When the culture was grown on agar media, the production of lysis was found to be associated only with the formation of a certain type of colony   The organism produced as a result of lysis two types of daughter colonies   one was similar to the mother colony and possessed continued capacity for lysis, the other did not lyse and was morphologically different from the first type   The lysing colonies possessed

strong proteolytic properties and apparently did not form any aerial mycelium, the nonlysing colonies were less proteolytic and formed a chalky aerial mycelium, which changed the reaction of litmus milk to alkaline   In broth cultures, lysis took place in 2 to 3 weeks, it was associated with the living organism and was of the nature of a nonenzymatic but nontransmissible lytic factor

Wieringa and Wiebols (1936) and Wiebols and Wieringa (1936) reported that various actinomycetes isolated from infected potatoes underwent lysis in culture This phenomenon was believed to be due to the production of specific transmissible phages   Various organisms yielded phages which were active also upon other organisms, thus 1 *bovis* produced a phage which was active upon 1 *scabies* and 1 *farcinicus*   These investigators were thus the first to emphasize the formation by actinomycetes of filterable and transmissible agents comparable to bacteriophages and polyvalent in nature   Their probable role in the control of potato scab in the soil has been suggested

In view of the taxonomic relationship between the actinomycetes and mycobacteria, it may also be of interest to recall that Steenken (1935) observed lysis among the latter   This did not however appear to be a result of phage action A virulent culture of *Mycobacterium* yielded a nonvirulent strain (R variant) which began to lyse after 3 or 4 months

Krassilnikov (1938) made a detailed study of the course of autolysis of different actinomycetes isolated from the soil   A well developed colony on an agar plate gradually became slimy flat, and transparent   When transferred to a fresh medium, the colony either failed to develop or produced a much-delayed growth Autolysis did not occur over the whole surface of the colony, but took place in sectors or spots frequently it began in the center and spread toward the periphery   This phenomenon appeared to be very general among parasitic organism and occurred less commonly among the saprophytes

Krassilnikov and Korenniako (1939) emphasized the resemblance of the process of autolysis among actinomycetes to the Twort phenomenon, or phage production by bacteria   They reported that the lytic factor of actinomycetes, contrary to the observations of Wieringa and Wiebols, was highly specific, since it had no action on other species or even on other strains of the same species of *Actinomyces*   Lysis took place when growth of the organism was delayed for one reason or another or at the time of aging of the culture   Since different cultures underwent lysis with varying degrees of rapidity, it was assumed that the quantitative production of the lytic factor or its mode of action was distinct with different organisms   At high temperatures (60 to 70 C), lysis occurred in a few minutes   The lytic agent was resistant to 80 C for 1 hour, but was destroyed at 100 C in 5 minutes   Not only living but also dead cells were affected, thus showing a difference in action from that of true phage

Katznelson (1940) isolated from manure composts a thermophilic culture of an actinomyces which underwent rapid lysis at 50 C when grown on starch ammonium sulfate agar media, no transmissible lytic agent could be demonstrated Schatz and Waksman (1945), studying the production of streptomycin by different strains of *S griseus* obtained from colonies of a given culture, observed

that colonies devoid of aerial mycelium produced no streptomycin Such colonies gave rise to cultures which underwent much more rapid lysis than the normal cultures producing aerial mycelium In the practical production of streptomycin it is generally observed that under submerged conditions of growth maximum formation or accumulation of the antibiotic corresponds to the beginning of lysis, advanced lysis usually results in a rapid destruction or inactivation of the streptomycin already produced

Although these meager series of observations seemed to point definitely to the capacity of some actinomycetes to produce phagelike agents under certain conditions of culture, they threw very little light upon the nature and activities of these agents They were not even sufficiently differentiated from lytic reactions due to enzymelike mechanisms

The problem of phage production by actinomycetes entered a new phase with the discovery that the streptomycin-producing strains of *Streptomyces griseus* are subject to attack by a virus or a phagelike agent This reaction appeared to be quite distinct from the lytic phenomenon mentioned above

Saudek and Colingsworth (1947) were the first to report the production by *S griseus* of a transmissible lytic agent which had all the properties of phage The phage developed in the presence of young cultures of *S griseus* These workers used the plaque method with a phage-sensitive strain of *S griseus* for measuring the concentration of the phage Streptomycin production was partly or completely prevented by the phage Cultures resistant to the phage could easily be isolated

This problem was independently investigated by Woodruff (1947) When a submerged culture of *S griseus* was placed in a stationary condition, with plugs removed from the flask, and exposed to laboratory air for 24 hours, the freshly formed pellicle showed evidence of plaque formation The same phenomenon was observed in a factory 500 miles away Multiplication of the phage took place upon each transfer of a filtered culture into a fresh culture of *S griseus* After six transfers, each phage particle increased to 75×10^{70} particles The phage was active against all streptomycin-producing strains of *S griseus* but not upon the non-streptomycin-producing strains The culture produced phage-resistant strains readily These retained the capacity of producing streptomycin but were not free from phage The actinophage had properties similar to bacterial phages, such as those of *Escherichia coli*, as shown both by cultural characteristics and by appearance in photographs made by means of an electron microscope

It has thus been established beyond doubt that at least certain species of *Streptomyces* can be attacked by a true phage In view of the possible importance of this phenomenon in streptomycin production, and also in order to throw light upon its significance in the life cycle of the organisms producing the phage and in the taxonomy of actinomycetes as a whole, a detailed study was undertaken of the production, nature, and activity of this phage Certain of the more immediate problems were at first investigated These included the sensitivity of various strains of *S griseus* to actinophage, the effect of actinophage upon the

growth and streptomycin production by *S griseus* in static and in submerged culture, multiplication of active phage under different conditions of culture of *S griseus*, effect of temperature upon phage activity, and the action of *S griseus* phage upon non-streptomycin-producing strains of this organism and upon other actinomycetes

EXPERIMENTAL METHODS AND RESULTS

Cultures used A number of strains of *S griseus* were used These included several original isolations of streptomycin-producing cultures and a number of active and inactive strains obtained from them by colony selection In addition, other strains of *S griseus* not producing any streptomycin and other actinomycetes taken from the culture collection or freshly isolated from various substrates were also investigated

The more important cultures are listed here

(1) Streptomycin producing strains of *S griseus*
   S griseus 3163, the original streptomycin producing culture 18-16
   S griseus 3180 an original culture isolated independently
   S griseus 3181 another original isolate
   S griseus nos 4 and 9 strains isolated from culture 3163
   S griseus 3475 a strain isolated from culture no 4
   S griseus 3523 and 3524 streptomycin producing cultures comparable to no 4 and 9
   S griseus 3475 2PR a phage resistant culture obtained from 3475

(2) Non streptomycin producing cultures of *S griseus*
   S griseus 3478, a culture producing grisein
   S griseus 3326 the original culture of *S griseus* isolated in this laboratory in 1915 and kept on artificial media since then
   S griseus 3326a the same culture as above, which was deposited with the Central bureau in Holland in 1920 and recently received from that collection
   S griseus 3522 culture isolated by Bucherer and also received from Holland
   S griseus 3495, culture isolated from no 4, it does not produce streptomycin but forms another still unidentified antibiotic

(3) Other cultures
   Streptomyces bikiniensis a streptomycin producing organism distinct morphologically and culturally from *S griseus* and isolated from a Bikini soil (Johnstone and Waksman 1947)
   Streptomyces violaceus ruber, a culture isolated from the soil and kept for many years in the collection

Phage used A phage preparation, originally obtained from Merck and Company and designated as M, was used in all these investigations

Assay methods In preliminary experiments, 0 1-ml portions of *S griseus* culture filtrate containing the phage were added to 10-ml portions of nutrient agar, these were poured into petri plates and allowed to solidify Aqueous spore suspensions of different strains of *S griseus* were streaked on the surface of the plates and incubated at 28 C for 18 hours The growth of strains nos 4 and 9 was completely inhibited Strain 3475 showed a few small colonies on the plate As these appeared to be resistant to the action of the phage, they were picked from the plates and inoculated upon fresh agar slants Several cultures were

obtained from the resistant colonies   One w is selected for further study and designated as 3475-2PR

To assay the phage preparations quantitatively, 10-ml portions of nutrient agar were poured into a series of five petri plates   These were streaked with spore suspensions of different strains of S griseus and incubated for 24 hours The diluted phage preparations were then poured over the plates   Upon further incubation at 28 C, the surface growth of the actinomyces streaks showed numerous plaques (figure 1)   These were difficult to count   The results obtained
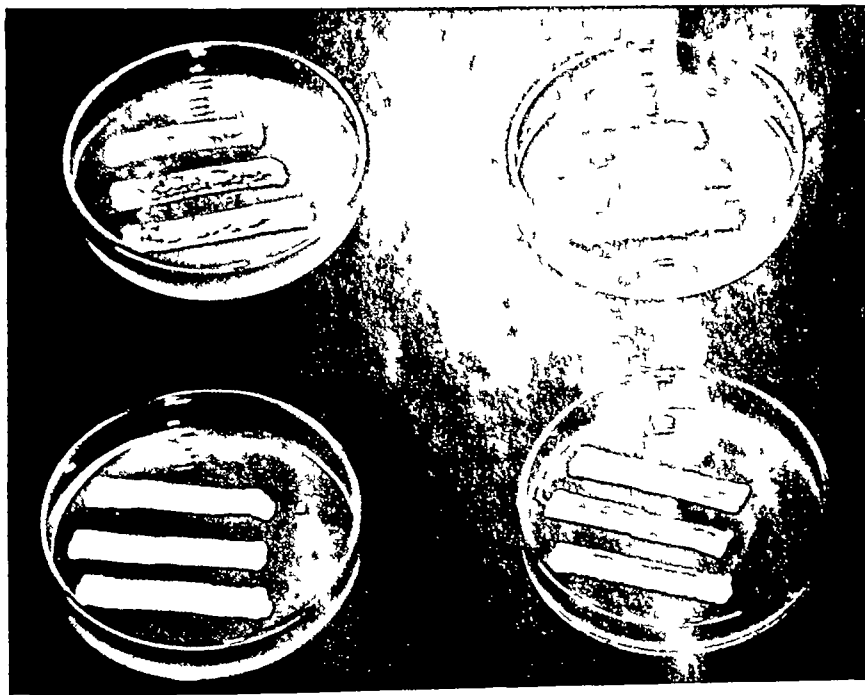
FIG 1 EFFECT OF ACTINOPHAGE UPON THE DEVELOPMENT OF DIFFERENT STRAINS OF S GRISEUS

Top Phage treated, bottom controls   Left pair 3475 2PR, right pair 3475

were only approximate and need not be reported here, since they were qualitative rather than quantitative

When the phage was added simultaneously with the inoculum to the fresh medium, growth of the organism was completely prevented, but when the phage was added to cultures which had already been well sporulated, no phage multiplication occurred   These results prove emphatically that the actinophage acts best upon young cultures, as already emphasized by others (Woodruff, 1947) Various streptomycin-producing strains of S griseus appeared to respond differently to the action of the phage, some being less affected than others When colonies were picked from the agar streak that had been infected with

phage, they produced cultures that were especially resistant to the action of the phage, although they were still capable of supporting considerable phage growth

The following method was finally adapted for assaying the concentration of phage in a given preparation   A 5-day-old shaken culture of a streptomycin producing strain of *S griseus* (no 3463 being used mostly for this purpose) was filtered aseptically through paper and used as the source of culture material for inoculation of plates   The phage preparation, designated as M-1, was obtained by inoculating the M phage into young cultures of *S griseus*, which were allowed to incubate for 24 to 72 hours and then were passed through a Seitz filter   A
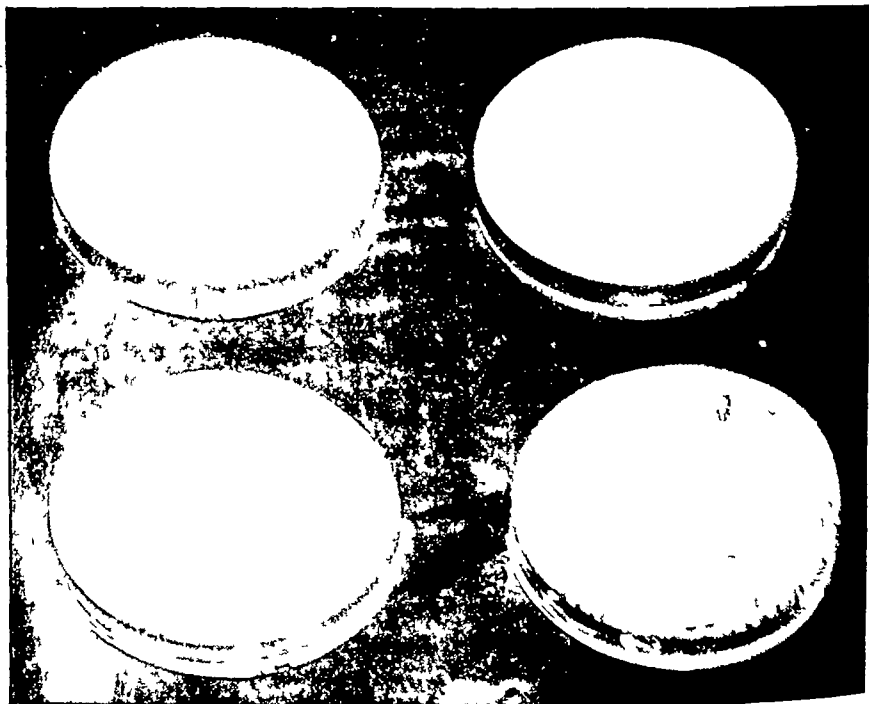
FIG 2 PLAQUE FORMATION BY ACTINOPHAGE ON PLATES OF S GRISEUS
Top left to right, control and phage diluted 1 100,000, bottom left to right, phage diluted 1 10,000 and 1 1,000

series of dilutions of phage, ranging from 1 10⁶ to 1 10¹², were added to 10 ml portions of sterile nutrient agar, which had been inoculated with 0 1-ml portions of the paper-filtered *S griseus* culture   The agar portions were poured into plates, mixed thoroughly, and incubated at 28 C for 2 days   The plaque counts were then made, as shown in figure 2   The M-1 phage preparation contained 4 2 × 10¹⁰ particles per ml

This method of assay gave accurate and reproducible results   The first phage preparation, M-1, was kept in the refrigerator and used as a standard   This procedure was now standardized, especially in regard to the effect of size of inoculum and of temperature of incubation   Three different amounts of filtered

7-day-old shaken culture of no 3463 were added to nutrient agar to give final 10, 1, and 0 1 per cent concentrations They were inoculated with different dilutions of M-1 phage and incubated at 28 C for 48 hours The following results were obtained

Culture inoculum per 100 ml of agar ml	Plaque counts × 10⁷
10 0	391
1 0	698
0 1	756

These results show that a lower inoculum gave higher counts, a 1 per cent inoculum was, therefore, adopted for all subsequent work

In studying the effect of temperature of incubation upon plaque development, two temperatures were used, 28 C and 37 C Normal plaque development took place at the lower temperature No plaques appeared on the plates incubated at 37 C When these plates were subsequently placed at room temperature for an additional 24 hours, plaques were rapidly produced with 1 per cent inoculum None of the plates inoculated with 10 per cent of culture material produced any plaques, which points to the fact that not only is a temperature of 37 C unfavorable for phage multiplication, but at that temperature an excess inoculum exerts a destructive, or at least an adsorptive, effect upon the phage

Effect of the phage on growth and streptomycin production by S griseus In a preliminary experiment on the effect of phage upon stationary cultures grown in standard medium for streptomycin production,[3] it was found that when the phage was placed in drops upon 2- and 3-day-old pellicles and allowed to incubate further at 28 C, many clear patches were produced in the pellicles, especially in the younger ones Further investigation indicated that the study of the effect of phage upon growth of S griseus and upon streptomycin production could best be conducted in submerged cultures This is brought out in table 1 When the phage was added at the time of inoculation of the cultures, very little streptomycin was produced at the earlier periods of incubation, namely, after 3 and 4 days When the cultures were allowed to incubate further, active streptomycin production occurred, as shown by the 5- and 6-day readings This is a result, no doubt, of the development of resistant strains in the culture upon continued incubation

A comparative study of the effect of phage on streptomycin production by strain 3475 of S griseus and by the phage-resistant culture 3475-2PR, isolated from the foregoing strain, under submerged and stationary conditions of growth, tends to confirm the observation above This is brought out in table 2 After 3 and 4 days' incubation in shaken cultures, no streptomycin was produced by the original strain in the presence of phage, however, after 8 days streptomycin production occurred, the activity of the low phage inoculum equaling that of the controls The resistant strain, on the other hand, gave good streptomycin

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[3] This medium contained 1 per cent glucose, 0 5 per cent each of peptone, meat extract, and NaCl in tap water

activity during the early incubation period, the presence of phage exerting only a slight depressive effect upon the total activity   Similar results were obtained in stationary cultures, the recovery of the streptomycin-producing capacity in the phage-containing cultures being much slower, however   Under these conditions, the phage-resistant strain did not produce so much streptomycin as the original culture

TABLE 1

Influence of phage on streptomycin production in submerged culture

PHAGE ADDED*	INCUBATION DAYS			
	3	4†	5	6†
	Streptomycin produced µg/ml			
0	21	100	99	133
+	5	8	7S	56

* One ml of phage, containing 4×10^{10} particles per ml, was added to 60 ml portions of glucose peptone meat extract NaCl medium in 250 ml Erlenmeyer flasks inoculated with spore suspensions of *S griseus*

† Cultures were kept static for 24 hours, then placed in a shaking machine

TABLE 2

Effect of addition of phage upon growth and streptomycin production by original S griseus and by a phage-resistant strain]

PHAGE ADDED		STREPTOMYCIN µG/ML PRODUCED AFTER DAYS					
After incubation	Phage per ml, $\times 10^7$	Submerged cultures			Stationary cultures		
		3	4	8	8	10	24
Strain 3475							
hours							
0	0	32	111	192	140	1S8	1S9
Start	0 7	<5	<5	20S	<5	<5	113
Start	70	<5	<5	128	<5	<5	93
24	70	<5	<5	122	<5	<5	24
Resistant strain 3475-2PR							
0	0	124	132	172	16	23	79
Start	70	117	10S	140	19	50	36

More detailed results of further experiments on phage multiplication and the effect of phage upon streptomycin production under submerged and stat   conditions of culture are reported in tables 3 and 4   When the phage was add to the culture simultaneously with the inoculum, it multiplied rapidly and first completely prevented streptomycin production, on further incubati streptomycin production set in rapidly, and later tended to approach that of control   The only possible interpretation is that the development of a .

resistant strain of *S griseus* occurred in the culture   When the phage was added to the submerged cultures 16 hours after inoculation, its rate of multiplication was much more rapid, because of the greater amount of available mycelium, with a corresponding reduction in streptomycin production   Here again, streptomycin production set in rapidly later, as a result of the development of resistant strains   Similar results were obtained when the phage was added to the

TABLE 3

Effect of phage upon the production of streptomycin by S griseus in submerged culture

PHAGE* ADDED AFTER	INCUBATION DAYS					
	3		5		7	
	Phage × 10⁸	Sm†	Phage × 10⁸	Sm	Phage × 10⁸	Sm
		μg/ml		μg/ml		μg/ml
Control	0	94	0	168	0	258
0 hours	1	<5	100	<5	208	192
16 hours	37	<5	75	5	92	172
2 days	177	22	222	18	382	36
4 days	—	—	24	122	45	272
6 days	—	—	—	—	53	300

* One tenth ml of phage preparation, containing 40×10^8 particles, was added to each flask containing 60 ml of medium, this is equivalent to $0\ 67 \times 10^8$ phage particles per 1 ml of medium   All results are reported per 1 ml of culture
† Sm = streptomycin

TABLE 4

Effect of phage upon the production of streptomycin by S griseus in stationary culture

PHAGE* ADDED AFTER	INCUBATION DAYS					
	9		13		17	
	Phage × 10⁸	Sm	Phage × 10⁸	Sm	Phage × 10⁸	Sm
		μg/ml		μg/ml		μg/ml
Control	0	306	0	252	0	273
0 hours	19	<5	31	16	24	178
16 hours	27	<5	38	6	33	79
2 days	47	26	588	36	605	94
6 days	29	300	38	219	73	303
12 days	—	—	0 9	185	0 4	303

* Same as table 3

48-hour-old cultures, the effect being magnified, as shown by the more rapid rate of phage development   The small amount of streptomycin formed at the time the phage was added did not increase until the seventh day, when the ability to form streptomycin was apparently recovered   When the phage was added to the 4- and 6-day-old cultures, at a time when growth had reached a maximum, there was a very limited amount of phage multiplication, and little effect was exerted on the streptomycin that had already been produced in the medium

The results obtained under stationary conditions fully confirmed the results on the submerged cultures, namely, that phage multiplication was at a maximum when added to the 2-day-old cultures, that the addition of phage at the time of inoculation or soon afterward represses streptomycin production, that this is

TABLE 5

Effect of phage upon the growth, phage multiplication, and streptomycin production by different actinomycetes in stationary cultures

ORGANISM	PHAGE ADDED*	9 DAYS		13 DAYS	
		Phage per ml $\times 10^7$	Sm	Phage per ml $\times 10^7$	Sm
			$\mu g/ml$		$\mu g/ml$
Streptomycin-producing strains of *S griseus*					
No 3463	0	—	—	0	21
	+	—	—	200	5
No 3475	0	0	30	0	180
	+	>50	<5	370	<5
No 3480	0	0	31	0	189
	+	10	<5	30	28
No 3481	0	0	73	0	174
	+	50	<5	260	13
No 4	0	0	43	0	201
	+	30	<5	160	<5
3475-2PR	0	>0 01	40	40	129
	+	>50	16	370	75
S griseus 3478	0	0	<5	0	<5
	+	0	<5	0	<5
S griseus 3326a	0	—	—	0	<5
	+	—	—	<0 2	<5
S bikiniensis	0	0	<5	0	30
	+	3	30	7	33

* Each 60-ml flask of culture received at start 0 1 ml of M-1 phage, amounting to 7 × 10⁷ particles per 1 ml of medium

followed by the development of resistant strains which result in a considerably delayed formation of the antibiotic, and that, when added to older cultures, some phage development occurs with little effect upon the streptomycin in the culture

Effect of phage M-1 upon different strains of S griseus and upon other actino cetes A detailed study of the effect of phage upon different cultures of actino cetes, comprising different species and strains, brought out the fact (tables 5

6) that phage M-1 affected all the streptomycin-producing strains of *S griseus*, it inhibited streptomycin production, and it multiplied at the expense of the growth of the organism   It had little effect upon the growth of other organisms The two non-streptomycin-producing strains of *S griseus* as well as some of the other actinomycetes tended to destroy or adsorb the phage, the mechanism of

TABLE 6

Phage multiplication in shaken cultures of various actinomycetes and its effect upon the production of antibiotics

ORGANISM	PHAGE* ADDED AFTER HOURS OF INCUBATION	TOTAL INCUBATION, DAYS				
		2	4		6	
		Phage per ml × 10⁷	Phage per ml × 10⁷	Antibiotic activity	Phage per ml × 10⁷	Antibiotic activity
				S units/ml		*S units/ml*
S griseus no 4	Control†	0	0‡	66	0	90
S griseus no 4	Start	22	650	35	930	48
S griseus no 4	24	9,500	7,000	96	4,600	135
S griseus no 4	48	—	166	120	90	90
S griseus 3478	Control	0	0	—	—	14
S griseus 3478	Start	8	1 3	—	—	15
S bikiniensis	Control	0	0	29	—	18
S bikiniensis	Start	0 05	0 13	24	0	30
S lavendulae	Control	—	0	15	—	<10
S lavendulae	Start	—	8 8	<10	—	<10
S violaceus-ruber	Control	—	—	—	0	—
S violaceus-ruber	Start	—	—	—	0 00002	—
Nocardia asteroides	Control	—	—	—	0	—
Nocardia asteroides	Start	—	—	—	9 4	—
Micromonospora sp	Control	—	—	—	0	—
Micromonospora sp	Start	—	—	—	9 1	—

* 70 × 10⁶ phage particles added per ml of culture
† *No phage was added to control cultures*
‡ One plaque appeared on one plate, there is some doubt as to whether this plaque was due to phage or was due to the growth of an *S griseus* colony that was antagonistic to the test organism

this reaction still being uncertain   The phage had no injurious effect either upon growth or upon streptomycin production of *S bikiniensis*

The foregoing results were confirmed by a number of other experiments, with minor variations   For example, no 3495, a strain of *S griseus* isolated from a streptomycin-producing culture, which does not form streptomycin but does form another antibiotic inactive against *Escherichia coli* but active against gram-positive bacteria, gave no phage multiplication but showed occasionally a

change in the nature of the antibiotic spectrum   *S bikiniensis* allowed no phage multiplication, or actually brought about the disappearance of the phage, and showed at times increased streptomycin production in the presence of the phage The latter reaction may have been due to nutritive effects of certain constituents of the phage preparation

In a series of experiments upon phage multiplication in cultures of different strains of streptomycin-producing *S griseus*, each of 10 such strains was inocu lated into four 60-ml portions of broth in 250-ml Erlenmeyer flasks   To two flasks of each series, 0 1-ml portions of phage M-1 were added at the time of inoculation, two flasks were left as controls   Phage determinations were made after 2, 4, and 6 days of incubation at 28 C   The antibiotic potency of the cul tures was determined by the usual cup technique against a streptomycin stand ard (table 7)

There was considerable variation among the different cultures both in the extent of phage multiplication and in the rapidity of recovery of streptomycin producing potency   As in previous experiments, the phage-resistant culture 3475-2PR showed comparatively little effect of the phage upon streptomycin production

Effect of temperature upon phage   In a preliminary experiment on the effect of temperature upon actinophage, several 5-ml portions of phage M-1, diluted to give 43 $\times$ 10^7 particles per ml, were placed in sterile test tubes and kept in a water bath at four different temperatures for 10 minutes   No significant de struction of the phage took place at 40 to 65 C, a definite reduction occurred at 75 to 80 C, and maximum destruction was reached at 100 C

In a more carefully controlled experiment, similar dilutions of phage were made   They were placed in water baths and incubated at various temperatures for 10 minutes and for 1 hour (table 8)   The phage was stable for 1 hour at 65 to 75 C   Appreciable reduction in the number of phage particles occurred in the tubes kept for 10 minutes at 85 to 90 C and a further decrease occurred upon continued incubation   Heating for 1 hour at 90 C was not sufficient, however, to destroy the phage completely

To determine the effect of temperature with prolonged storage upon the survival of phage, several 10-ml portions of phage M-1 diluted 1 100 with sterile water were added to test tubes, stoppered with sterile rubber stoppers, and placed at four different temperatures   After several periods of incubation phage determinations were made   The results show (table 9) that incubation for 3 days at 56 5 C brought about an appreciable decrease in phage concentra tion, after 12 days at this temperature more than 99 per cent of the phage was destroyed   At 37 C the decrease was much slower, incubation for 12 days giving about 58 per cent loss of phage concentration and nearly complete after 29 days   At 28 C there was a small decrease after 12 days' incubation marked decrease after 29 days   There was no change in concentration of phage at 6 C on continued incubation

Further studies on the effect of temperature upon phage multiplication firmed the previous results   The optimum was at 28 C   There was no in

in phage content at 37 C, and at 56 5 C more than 97 per cent of the phage was destroyed in 1 day   The extent of phage multiplication depended largely upon the size of the inoculum   The greater the number of cells of *S griseus* present in the culture, the greater was the amount of phage produced   At 56 5 C, the size

TABLE 7

Multiplication of phage in shaken cultures of streptomycin-producing strains of S griseus

STRAIN NO	ADDITION OF PHAGE	DAYS OF INCUBATION			
		2		4	6
		Phage* per ml × 10⁷	Sm	Sm	Sm
			μg/ml	μg/ml	μg/ml
No 3463	0	0	17	90	116
No 3463	+	84	<5	<5	34
No 3464	0	0	<5	28	20
No 3464	+	70	<5	<5	18
No 4	0	0	<5	15	12
No 4	+	15	<5	<5	<5
No 9	0	0	<5	>50	51
No 9	+	64	<5	<5	51
No 3475	0	0	<5	64	92
No 3475	+	83	<5	<5	76
No 3498	0	0	23	49	78
No 3498	+	76	<5	<5	16
No 3499	0	0	<5	43	90
No 3499	+	20	<5	<5	<5
No 3523	0	0	21	114	149
No 3523	+	81	<5	<5	20
No 3524	0	0	17	57	72
No 3524	+	146	<5	7	32
No 3475-2PR	0	<0 001	<5	98	104
No 3475-2PR	+	8	<5	42	104

* The cultures treated with phage contained at start 4 × 10⁷ phage particles per ml

of inoculum also had an effect upon the extent of phage destruction   the larger the inoculum, the lower was the rate of phage destruction   The nature of the medium in which the phage was suspended had a marked influence upon the rate of its destruction at 56 5 C   The phage suspended in water showed only about 40 per cent destruction in 1 day, whereas the phage placed in broth lost more than 99 per cent of its activity in the same time   After 3 days, the phage

diluted with broth was completely destroyed, whereas considerable phage was left in the aqueous suspension, although marked destruction had taken place

Multiplication of actinophage in the presence of living and dead cells of S griseus Finally, studies were made upon the ability of the phage to multiply at the ex

TABLE 8

Effect of temperature upon the stability of actinophage

(At start, 43×10^7 phage particles per ml)

TEMPERATURE	PHAGE $\times 10^7$ PER ML, AFTER	
	10 minutes	1 hour
C		
Control	43	
65	45	45
75	—	45
85	0 07	0 0003
90	0 001	0*

* The actual count was 5 particles per ml

TABLE 9

Stability of phage in aqueous suspension upon storage at several temperatures

TEMPERATURE OF STORAGE	PHAGE PARTICLES $\times 10^7$ PER ML AFTER STORAGE*		
	3 days	12 days	29 days
C			
6	44	—	60
28	31	20	0 00005
37	37	15	0 0000009
56 5	18	0 001	0

* At start all preparations contained 36×10^7 particles of phage per ml

TABLE 10

Multiplication of actinophage in living and dead cultures of S griseus

S GRISEUS CULTURE	PHAGE $\times 10^7$		
	Start	1 day	2 days
Living	43	136	189
Dead	65	76	58

pense of living and dead cultures of *S griseus* Two 10-ml portions of a 40 hour old shaken culture of a streptomycin-producing strain (no 3475) were transferred aseptically to sterile test tubes   One tube was placed in a water bath at 75 C for 10 minutes, to kill the spores and mycelium of *S griseus*, the second tube was not heated   To both tubes were added 0 1-ml portions of phage M-1, the tubes were incubated at 28 C and phage concentrations determined at the start and

after 1 and 2 days   The results presented in table 10 show that no multiplication of the phage took place in the presence of dead cells of *S grisaus*, in the presence of living cells, a fourfold increase in phage concentration occurred

SUMMARY

The results obtained by Saudek and Colingsworth and by Woodruff on the production of phage by streptomycin-producing cultures of *Streptomyces griseus* have been fully confirmed

Certain cultures of *S griseus* are subject to attack by a virus which can be designated as "actinophage"

This phage attacks only the streptomycin-producing strains of *S griseus*, it has no effect on other streptomycin-producing organisms

In cultures of strains of *S griseus* that do not produce streptomycin, the phage does not multiply and may actually be destroyed or adsorbed

The actinophage of *S griseus* multiplies only at the expense of living cell material and not upon the heat-killed material of this organism

Phage-sensitive cultures of *S griseus* give rise rapidly to strains which are resistant to the action of the phage

The actinophage has an optimum temperature for multiplication at 28 C It does not multiply at 37 C or above

Actinophage can withstand a temperature of 75 C for 1 hour, but is completely destroyed at 100 C in 10 minutes

The actinophage can be stored at 6 C without loss of activity, but storage at 28 C or at higher temperatures results in a loss of activity, the rate of loss being proportional to the temperature

The nature of the medium in which the actinophage is suspended greatly influences the rate of its destruction

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TWO STREPTOMYCIN-RESISTANT VARIANTS
OF MENINGOCOCCUS[1]

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One of the striking differences between penicillin and streptomycin is the rapidity with which microorganisms develop resistance to the latter   Resistance to penicillin can be acquired, but always in relatively small increments at each subcultivation on artificial media or in each passage of the strain through an experimental animal   A high degree of resistance can be attained *in vitro* or *in vivo* only by repeated exposure to increasing concentrations of the drug

Miller and Bohnhoff (1947a), for instance, found that 147 transfers onto media containing increasing concentrations of penicillin raised the resistance of a strain of meningococcus sufficiently to permit it to grow abundantly on media containing 5,000 units per ml   They have also shown (Miller and Bohnhoff, 1946b, 1947b) that the resistance of a stra n of meningococcus could be increased by serial passage through m ce treated with subcurative doses of penicillin   They used cultures of hearts' blood as inocula for each succeeding animal passage   The dose required to protect approximately half of the mice rose from 10 units to 1,700 units in the course of 61 passage inoculations

Resistance to streptomycin, on the other hand, was found to develop with such rapidity that two or three transfers onto media containing increasing concentrations sufficed to permit meningococcus or gonococcus to multiply on media containing 50,000 μg of streptomycin per ml (Miller and Bohnhoff, 1946a)   Meningococci which were rendered streptomycin-resistant by this means retained approximately the virulence of the original parent culture and were resistant to streptomycin *in vivo*   Mice inoculated with such resistant meningococci died in spite of doses of streptomycin which would have protected them against infection with normal meningococci

The present communication presents evidence that this rapid development of streptomycin resistance by meningococcus is due to the selective propagation of resistant variants which become apparent during growth on streptomycin-containing media   These variants are presumed to originate from streptomycin-resistant mutants which are arising regularly in the bacterial population of the normal parent strain before its exposure to the drug   In the course of these experiments, a second variant has been encountered which is not only resistant to streptomycin but is actually dependent on streptomycin for its growth

in vitro and *in vivo*   Both variants have developed from each of 18 strains of meningococcus [2]

Strains of meningococcus   The 18 strains used in these experiments included (a) old stock strains which have been under cultivation in the laboratory for several years, (b) strains recently isolated from cases of epidemic meningitis, and (c) strains isolated from the nasopharynx of healthy carriers   Each of the strains was definitely identified as a member of one of the 3 fixed types I, II, or II alpha   All of the strains produced colonies typical of meningococcus and fermented only glucose and maltose

Media   The medium used most commonly throughout these experiments has been described in a previous communication as casein digest agar (Miller and Bohnhoff, 1947a)   Several other media were employed at various times for purposes of comparison and to make certain that the results obtained were not dependent on any ingredient of the medium itself   Media thus employed were meat digest cysteine agar (Miller and Bohnhoff, 1947a), Difco nutrient agar, brain heart infusion agar, and proteose peptone no 3 agar

The media were usually enriched by the addition of fresh, defibrinated sheep or rabbit blood   A few experiments were conducted with agar containing rabbit serum

When a liquid medium was required trypticase soy broth[3] was used

Preparation of streptomycin media   A total of 25 preparations of streptomycin[4] have been used   They were obtained from seven manufacturers and varied widely in streptomycin activity but included some preparations of an especially high degree of purity

Plates of streptomycin agar were made up as follows and were always used within a few hours of preparation   a saline solution of streptomycin was diluted to convenient concentrations and 1 ml of appropriate dilutions pipetted into each petri dish   Five-tenths ml of fresh, defibrinated blood were then put beside it   Melted agar (cooled to 45 C) was added, and the contents of each plate were thoroughly mixed

Method of inoculation of the streptomycin plates   When heavy seedings of meningococci were to be planted onto a series of plates containing graded concentrations of streptomycin, the following technique was employed because it distributed the inocula evenly and did not break the surface of the agar

After the agar had set, 5 small glass balls (about 6 mm in diameter), such as

[2] Our preliminary communication (Miller and Bohnhoff, 1947c) reported that these variants developed from 16 of 18 strains   The 2 strains originally considered failures have been re-examined and found to produce small numbers of both A and B variants

[3] Baltimore Biological Laboratory

[4] Preparations of streptomycin were supplied by the Antibiotics Study Section of the National Institute of Health, U S Public Health Service, the Division of Penicillin Control and Immunology, Food and Drug Administration, Abbott Laboratories, Commercial Solvents Corporation, Eli Lilly & Company, Merck & Co , Chas Pfizer & Company, E R. Squibb & Son, and Upjohn Company

are customarily used for defibrinating blood, were placed on the surface of the agar in each plate. It was found to be convenient to have these "beads" distributed in test tubes, 5 to a tube, before sterilization, so that the whole contents of a tube could be rolled out gently onto the agar surface.

The 18-hour growth from an agar culture in an ordinary 16-ounce medicine bottle was harvested in 9 0 ml of gelatin Locke's solution,[5] sedimented by centrifugation, and resuspended in 0 5 ml gelatin Locke's solution. The meningococci were dispersed by drawing the suspension repeatedly into a capillary pipette from which one drop was allowed to fall onto the agar in each plate. These inocula contained approximately 1 0 to 2×10^{10} microorganisms. The plates were then stacked in a holder and shaken gently in all directions so that the beads rolled back and forth over the surface of the agar and distributed the inocula uniformly. The beads were then discarded. The plates were incubated for 3 days, the first in a candle jar, and then allowed to stand for a few more days at room temperature. They were all examined carefully each day for 5 or 6 days.

Mouse inoculations were made to determine virulence and also streptomycin resistance. A loopful of growth from an 18-hour culture was rubbed up in a few ml of gelatin Locke's solution and the suspension diluted until it reached a density equal to no 3 of the McFarland series (Kolmer and Boerner, 1945), which experience has shown to contain approximately one billion meningococci per ml. From this standard suspension, 10-fold dilutions were made in 4 per cent mucin[6] and 1 ml quantities injected intraperitoneally into mice weighing 16 to 20 grams (Miller and Castles, 1936).

Mice were treated with streptomycin by the injection of the desired dose in 0 5 ml of saline under the skin of the animal's back.

As many as possible of the mice that died were autopsied, and cultures of hearts' blood were made on casein digest agar and also on the same agar to which 100 μg of streptomycin per ml had been added.

EXPERIMENTAL RESULTS

The two variants described below appeared when meningococci were inoculated onto media containing streptomycin greatly in excess of that which is considered the optimal bactericidal concentration. Identical results were obtained from cultures started with a single isolated colony and from an ordinary transfer of a stock culture. A heavy seeding of an overnight growth of a normal, sensitive strain of meningococcus was planted onto a series of 8 to 12 plates containing graded concentrations of streptomycin. As most of the experiments were performed with one preparation of streptomycin,[7] the concentrations given below are those of that single preparation. The range varied from 10 μg per ml to 10,000 μg per ml. The intermediate concentrations were usually 20, 40, 60, 100, 200, 400, 600, 1,000, and 4,000 μg per ml.

The growth on a series of 6 plates is shown in figure 1.

[5] Locke's solution containing 0 1 per cent gelatin

[6] Granular mucin, type 1701 W, supplied by the Wilson Laboratories, Chicago, Illinois

[7] A preparation marketed for therapeutic use by Eli Lilly & Co
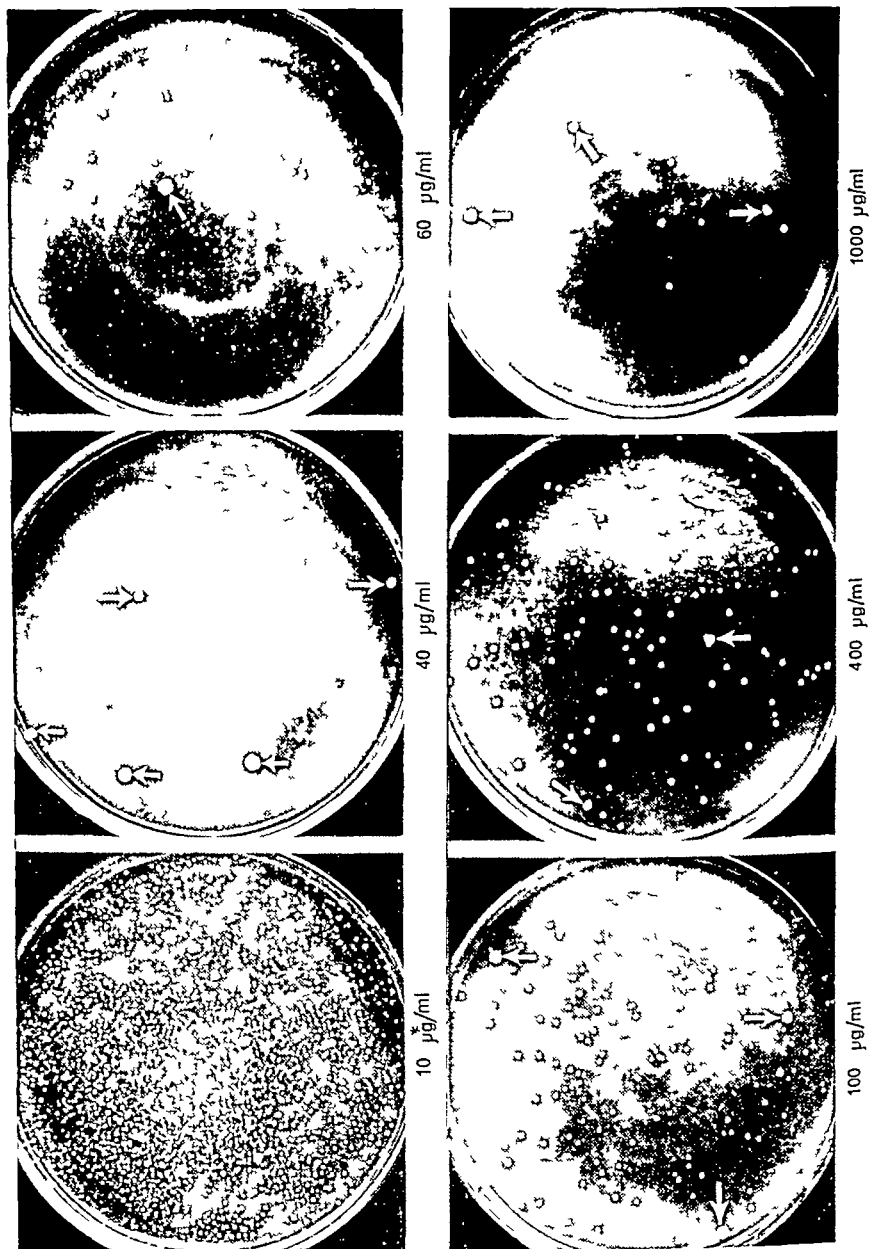
Fig 1 Growth of Meningococcus from Equivalent Inocula on Graded Concentrations of Streptomycin
Photographed after 72 hours incubation

The first plate (10µg) shows only normal meningococcus colonies   The type A colonies are indicated by arrows in third (60 µg), fourth (100 µg), and fifth (400 µg) plates as ow numerous type B colon as   The sixth plate (1000 µg) th wh fx) tho 1 on

* µg= Micrograms

10 *µg/ml

40 µg/ml

60 µg/ml

100 µg/ml

400 µg/ml

1000 µg/ml

After 24 hours' incubation, the plate containing 10 μg per ml showed confluent growth, and the one containing 20 μg per ml a very large number of colonies indistinguishable from normal meningococcus colonies A few of these normal colonies occasionally appeared on 40 μg per ml, but none on concentrations higher than that

Type A variant On plates containing 40 μg per ml, a second type of colony was visible at the end of 24 hours' incubation and continued to grow for the next 48 hours, reaching a size of 3 to 5 mm in diameter, i e , considerably larger than normal meningococcus colonies It differed from normal colonies in color as well as size, for it acquired a distinctly yellowish tinge which became more marked during the second and third day of incubation and after another day or two at room temperature This variant, which developed from each of the 18 strains, has been designated type A

Except on plates containing 10 to 20 μg per ml which were so crowded with normal colonies that they could not be distinguished, type A variants developed in about equal numbers from any given strain on all concentrations of the drug This number, however, varied from strain to strain Most strains produced 2 to 5 colonies per plate, an average incidence of approximately 1 to 3 in 10^{10} of original bacterial population Figure 2 presents the results of 32 experiments performed with one strain (113) and illustrates the uniformity of incidence of type A colonies One strain, however, developed greater numbers of type A colonies, 5 to 30 per plate

The type A variants had the following properties They were highly resistant to streptomycin as they were able to grow on concentrations of the drug as high as 10,000 μg per ml They were also able to multiply on streptomycin-free media They retained all of the following properties of the parent strains from which they arose morphology, staining characteristics, sugar fermentation, virulence for mice, and type specificity as determined by agglutination and by mouse protection tests Their streptomycin resistance was demonstrated *in vivo* by inoculating mice with mucin suspensions and treating the animals with 15,000 μg in 3 subcutaneous injections of 5,000 μg each at 1, 3, and 5 hours after inoculation The mice regularly died of meningococcal sepsis, and type A variants were cultured from their hearts' blood

No loss of streptomycin resistance has been detected in the type A variants either during passage through mice or during subcultivation on streptomycin-free media Two strains have been transplanted every 5 to 7 days for one year

Type A variants were found to be slightly more sensitive to penicillin than the parent strain from which they arose

Type B variants After 48 hours of incubation a second type of variant appeared on all concentrations of streptomycin above 40 μg per ml After another 24 hours' incubation, additional colonies of this type developed on concentrations of 60 and 100 μg per ml, but no new colonies appeared after 72 hours The size and color of these colonies varied with the concentration of streptomycin on which they grew On plates containing 60 to 100 μg per ml, they were very small and light gray, on concentrations above this range, they

were larger and had a distinctly yellowish tinge   On concentrations greater than 400 μg per ml, they resembled the type A colonies in size and pigmentation   The identification of doubtful colonies was made by subcultivation onto streptomycin free and streptomycin-containing agar

The number of type B colonies which developed from one type I strain (113) are plotted in figure 3, which shows (a) that the actual numbers varied considerably in different experiments and (b) that they were always most numerous between concentrations of 100 and 400 μg per ml   Curves of the numbers of colonies in individual experiments differed in height but almost always had the shape of the curve of the mean shown in figure 3

The meningococci composing these type B colonies had the following properties   They were resistant to streptomycin, for they were able to grow on concentrations as high as 5,000 μg per ml   They were dependent on streptomycin for growth, that is, they would grow abundantly from small inocula on concentrations between 100 and 400 μg per ml and would also grow from large inocula on concentrations as low as 5 μg per ml, but they could not be subcultured on media
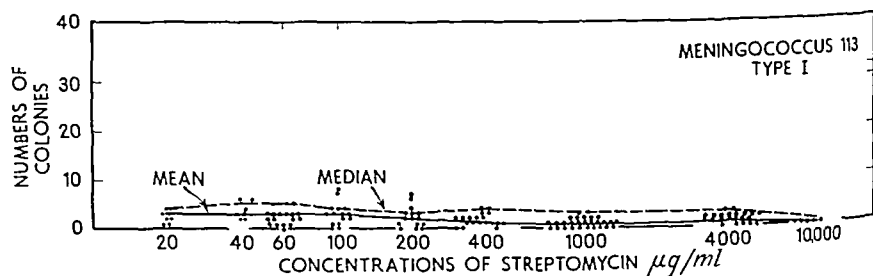
FIG 2 NUMBERS OF COLONIES OF TYPE A VARIANTS DEVELOPING ON GRADED CONCENTRATIONS OF STREPTOMYCIN

containing less than that minimum of streptomycin   They were nonvirulent for mice unless the mice were treated with streptomycin as described below   They were gram-negative and fermented glucose and maltose when the test media contained 100 μg of streptomycin per ml   They retained the type specificity of the parent strain from which they arose   Rabbit sera prepared against the parent strain conferred protection against experimental infection with these variants in mice treated with streptomycin

Microscopically, the type B organisms varied somewhat with the concentrations of streptomycin on which they had developed   Preparations made from the small gray colonies grown on 60 or 100 μg per ml showed them to be slightly larger than normal meningococci   Type B organisms growing on higher concentrations in larger pigmented colonies were indistinguishable from normal meningococci   This difference may well be related to the stimulating action of streptomycin mentioned below

Although the colonial development and microscopic appearance of type B variants differed according to the concentration of streptomycin on which they grew, the identity of all members of this variant was indicated by the following observations   When a type B variant was taken from any concentration and

subcultured onto another concentration, it always grew in colonies of the type regularly produced on that particular concentration   In other words, small gray colonies always developed on concentrations of 60 to 100 μg per ml and large colonies tinged with yellow on concentrations above 200 μg per ml, regardless of the concentration from which the inocula were taken
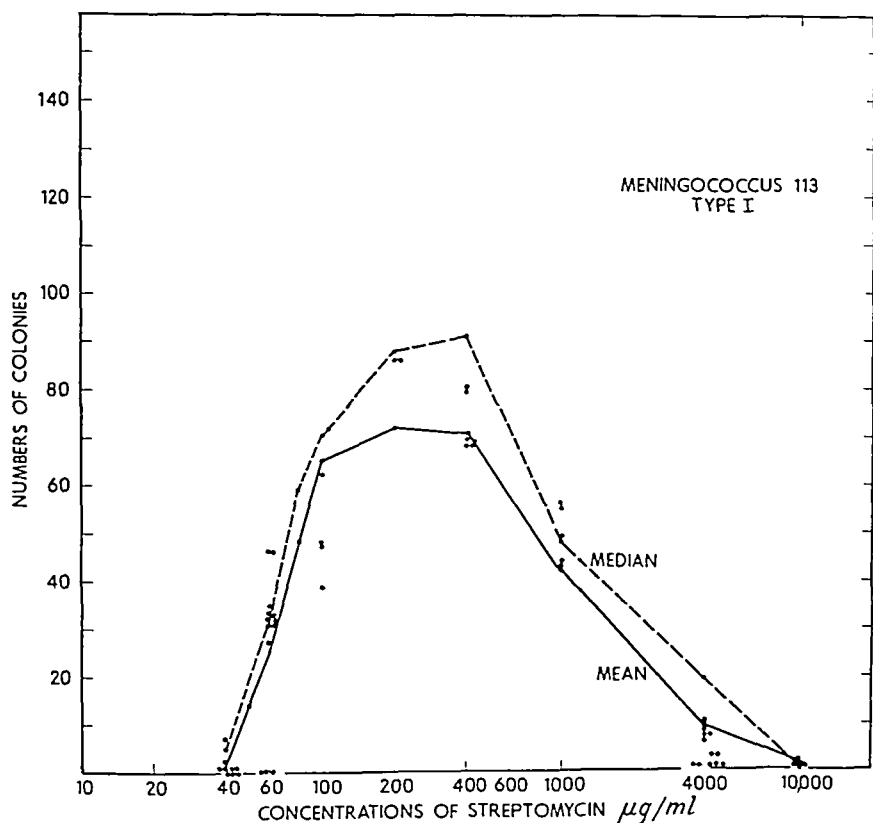
FIG 3 NUMBERS OF COLONIES OF TYPE B (STREPTOMYCIN-DEPENDENT) VARIANTS
DEVELOPING FROM HEAVY SEEDINGS ON GRADED CONCENTRATIONS OF
STREPTOMYCIN

Results of 35 experiments with meningococcus 113   The individual inocula contained approximately 1 0 to 2 0 × 10¹⁰

The dependence of type B variants on adequate concentrations of streptomycin for growth was also demonstrated by subculturing them into broth containing graded concentrations of the drug (see figure 4)   It will be seen that no growth occurred in the broth containing the low and high concentrations of streptomycin   The optimum range for multiplication in liquid media, therefore, approximated that for solid media

When a series of plates containing graded concentrations of streptomycin was planted with a pure culture of the B variant in small but equal inocula, the numbers of colonies which developed bore exactly the same relationship to

concentrations of the diug as did the B variants developing from the original inoculations with heavy seedings of the normal, parent strain   These experiments were made as follows   A suspension of B variant was prepared and diluted to a density equal to no 3 in the McFailand series, which experience has shown to contain about one billion meningococci per ml   This suspension was further diluted a millionfold and a drop (containing 35 to 50 meningococci) planted, by
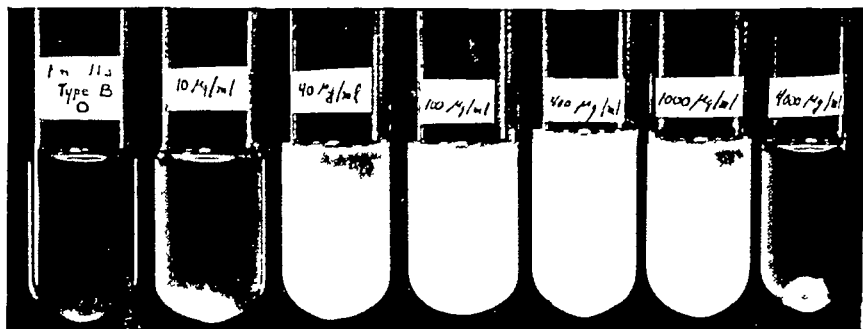
FIG 4   GROWTH OF TYPE B VARIANTS OF MENINGOCOCCUS IN BROTH CONTAINING GRADED CONCENTRATIONS OF STREPTOMYCIN
Left to right   Tube 1, control   Tubes 2 to 7 contain streptomycin—10, 40, 100, 400, 1,000, 4,000 µg per ml   The tubes were slanted sufficiently to afford a maximum increase in the surface of the broth and incubated for 24 houis

TABLE 1

Number and appearance of colonies developing from small, equal inocula of pure culture of type B variant

STREPTOMYCIN	NUMBER OF COLONIES	DESCRIPTION
µg per ml		
0	0	—
10	0	—
40	3	small, gray
60	8	medium, gray
100	33	medium to large, gray to slightly yellowish
200	35	large, slightly yellowish
400	30	large, yellowish
1,000	25	large, yellowish
4,000	6	small medium, yellowish
10,000	0	—

the method described above, onto a series of plates containing varying concentrations of stieptomycin   A poition of the inocula undoubtedly adhered to the beads and was iemoved with them   The iesults of a typical experiment are presented in table 1   It shows that the number and appeaiance of colonie developing on each concentiation resemble the number and appearance of type B variants which developed on those concentiations fiom the heavy seedings made originally with the parent strain   The homogeneity of the cultuie was estab-

lished by the fact that a number of colonies from each plate transferred onto streptomycin-free and streptomycin-containing agar grew only on the latter

Sensitivity of type B colonies to penicillin When type B colonies were tested for their sensitivity to penicillin, their growth was inhibited by approximately the same concentrations that inhibited the growth of the normal parent strain from which each variant arose They appeared therefore, to be as sensitive as normal meningococci to penicillin It should be pointed out however, that the tests could not be made on the same media because of the necessity of providing sufficient streptomycin for the development of type B variants in amounts which were bacteriostatic for the normal strain

Reversion of type B variants The type B variants continued to exhibit all the characteristics described during repeated subcultivation on streptomycin agar Their dependence on the drug has been complete except for four instances in which a single colony has developed on streptomycin-free agar The four exceptions were the only ones to occur among many subcultivations onto streptomycin-free agar In each instance the colony grew out slowly, but thereafter multiplied readily on streptomycin-free media They retained all of the properties of meningococci and are regarded as mutations back toward normal Then reversion to normal was not quite complete, however for three of them developed no type B variants when planted onto graded concentrations of streptomycin, but only type A The other reverted strain was able to develop both type A and type B colonies, but the numbers of the former were greater than those produced by its original parent strain It is clear, therefore, that none of these reverted mutants had regained all of the potentialities of the parent strain from which they were originally derived

Effect of inactivated streptomycin The type B variants were unable to grow on media containing streptomycin inactivated by hydroxylamine hydrochloride according to the method of Donovick, Rake, and Fried (1946) or by cysteine hydrochloride according to the method of Denkelwater, Cook, and Tishler (1945)

Experimental infection with type B variants The dependence of the type B variants on streptomycin for their multiplication could be demonstrated *in vivo* as well as *in vitro* When mice were inoculated with mucin suspensions of type B variants, the mice usually survived unless they were treated with streptomycin An occasional mouse died if very large inocula were used but meningococci were rarely recovered from its heart's blood, and then only on streptomycin-containing agar

On the other hand, mice treated with adequate doses of streptomycin usually succumbed to meningococcal sepsis, and type B variants were regularly recovered from cultures of their hearts' blood on streptomycin-containing agar Although hearts' blood was always planted onto streptomycin-containing and streptomycin-free media, no meningococci ever grew out on the latter

In table 2 are presented the results of a typical experiment in which mice were inoculated with 10^8 or 10^7 type B variants Streptomycin was administered subcutaneously 3 hours after infection in doses of 5 50, 500 5 000, and 10 000

μg, the last given in 2 doses of 5,000 each, the second dose $\frac{1}{2}$ hour after the first
It will be seen that all of the untreated controls survived and that the mortality
rose as the dose of streptomycin increased up to the largest dose   One or more

TABLE 2

Effect of streptomycin treatment on infection with type B variants

STREPTOMYCIN TREATMENT 3 HR AFTER INFECTION	APPROXIMATE NUMBERS OF MENINGOCOCCI INOCULATED					
	100 000 000			10 000 000		
	Result	Blood cultures		Result	Blood cultures	
		Strep free media	Strep * media		Strep free media	Strep * media
None	S			S		
	S			S		
	S			S		
	S			S		
5	21†	0	+	S		
	S			S		
	S			S		
	S			S		
50	21	0	+	S		
	90	—	—	S		
	S			S		
	S			S		
500	21	0	+	21	0	+
	21	—	—	21	0	+
	23	0	+	S		
	90	—	—	S		
5,000	21	—	—	23	0	+
	21	—	—	29	0	+
	21	—	—	49		
	27	0	+	S		
10,000 (2 doses of 5,000 ea)	21	0	+	21	0	+
	21	0	+	29		
	21			46		
	S			S		

+ = positive for meningococci, 0 = negative for meningococci, — = not cultured, S
= survived

* Streptomycin media = media containing 100 micrograms of streptomycin per ml
† Figures = hours of death

mice in each group were autopsied and cultures of their hearts' blood made on
streptomycin-free and streptomycin-containing agar   In every case meningo-
cocci were recovered on the latter but not on the former   The meningococci
recovered from the hearts' blood cultures had all of the characteristics of B

variants, that is, they retained their type specificity and their ability to ferment glucose and maltose but required streptomycin for growth

Unusually large inocula were used in the experiment just described   It has not as yet been possible, however, to produce fatal meningococcal sepsis regularly in mice with inocula smaller than 10^4 meningococci even though the animals received multiple injections of streptomycin   The virulence of the type B variants appears, therefore, to be less than that of the type A variants

<div align="center">DISCUSSION</div>

In these experiments two variants have arisen from cultures of meningococcus planted in heavy seedings onto a series of plates containing graded concentrations of streptomycin   It should be emphasized that each experiment was begun with a culture which had never been exposed to streptomycin and that inoculation onto the various concentrations of streptomycin was made at one time   Both variants developed from all of the 18 strains of meningococcus studied   They were gram-negative diplococci which retained the characteristic sugar fermentation and type specificity of the parent strains from which they arose   Both variants were highly resistant to streptomycin

One variant, designated type A, grew in large, yellowish colonies which appeared in approximately equal numbers on all concentrations of the drug although the numbers varied considerably from strain to strain   Its resistance to streptomycin was demonstrated *in vivo* as well as *in vitro*, for it produced infection in mice which proved uniformly fatal in spite of the administration of maximal doses of streptomycin tolerated by the mice

The incidence of the other variant, designated type B, as well as the size and color of its colonies depended on the concentrations of streptomycin onto which the original seedings were planted   Nevertheless, all of the B variants derived from any strain were found to be genetically alike

The striking characteristic of this variant was its dependence on streptomycin for multiplication on solid and in liquid media and in the body of an animal host The animal experiments indicated that this variant was nonvirulent for mice unless the animals were treated with adequate doses of streptomycin and that the dependence on streptomycin for growth persisted during and after multiplication within the body of the infected animal

It is impossible at the present time to be certain whether the substance required for the growth of the type B variant is streptomycin itself or some impurity which has been present in all of the preparations we have used   These numbered 25 and were obtained from seven manufacturers   Two of the preparations were described as being of an especially high degree of purity   It should be noted that streptomycin inactivated by hydroxylamine or by cysteine failed to support growth of the type B variants   This aspect of the problem is under investigation

The origin of these variants is difficult to explain unless one assumes that they both arise by current mutation, i e , from mutants which are constantly appearing in the original bacterial population of the parent strain   The type A

variants developed from any given strain with about equal frequency on all concentrations of streptomycin, although the frequency varied from strain to strain

The incidence of the type A variants from most strains was estimated to average 1 to 3 in 10^{10} of original bacterial population   One strain produced about 3 to 30 in 10^{10}

The maximum incidence of the type B variants varied from 2 to 15 per billion meningococci in the parent culture

When the type B variants were first observed, they were thought to arise by mutation which was induced by streptomycin   Subsequent observations have failed to support this hypothesis and have tended instead to indicate that, like the A variants, they, too, originated from mutations which were occurring regularly in the parent bacterial population

The fact that they appeared only on the high concentrations and only in greatest numbers within a certain range was explained by the demonstration that this range of concentrations was optimal for their development   Pure cultures of the B variants developed colonies on each concentration of streptomycin in the same relative numbers as did heavy seedings of the parent strain from which they arose   The greater proportion of them were able to reproduce only on certain concentrations, above and below that optimal range few or none developed   In other words, the type B variants developed approximately the same number of colonies on each of a series of concentrations whether they were planted in pure culture or together with myriads of normal, streptomycin sensitive meningococci   This observation seems to indicate that the streptomycin requirement of the type B variants for their multiplication is quantitative as well as qualitative

The variation in size and color of colony of the B mutants can only be attributed to the direct effect of streptomycin on the physiology of the microorganisms   Benham (1947) found that streptomycin increased the oxygen uptake of a normal strain of typhoid bacilli but not of a resistant one unless high concentrations of the drug were used

Several studies on the development of streptomycin resistance have appeared Chandler and Schoenbach (1947) for staphylococcus, streptococcus, and pneumococcus, Hamre, Rake, and Donovick (1946) for *Klebsiella*, and Klein and Kimmelman (1946a, 1946b)   Alexander and Leidy (1947), using a technique similar to ours, isolated streptomycin-resistant variants from *Hemophilus in fluenzae* and estimated their incidence as 1 in 1 1 billion to 1 in 13 8 billion members of the original bacterial population   As none of these authors mentions dependence on streptomycin as a characteristic of the resistant strains, one must conclude that they were dealing with resistant variants analogous to the type A variants herein described   It is quite certain that the streptomycin-resistant gonococci and meningococci reported earlier by Miller and Bohnhoff (1946a) were type A variants

Hall and Spink (1947) describe a strain of *Brucella* which became highly resistant to streptomycin   This strain was recovered from the blood stream of a

patient with *Brucella* endocarditis and had apparently developed a considerable degree of resistance *in vivo* After it had become highly resistant, it produced two types of colonies, a large one, which grew rapidly, and a small one, which grew slowly Although this latter variant was able to grow on streptomycin-free agar, it grew better on media containing 50 to 100 μg of streptomycin per ml It is possible that this second type of colony is similar to our B variant

It should be noted that Welch, Price, and Randall (1946) were able to demonstrate large numbers of viable typhoid bacilli in broth cultures containing streptomycin in concentrations greater than the minimum which inhibited growth They also found that the mortality rate of mice infected with typhoid bacilli was increased by treatment with small doses of streptomycin (0 05 to 1 0 μg) The mortality rate, however, was decreased when larger doses were administered

For a comprehensive discussion of the general problem of bacterial mutation, the reader is referred to the recent review by Luria (1947)

Studies on the growth requirements of mutants isolated from cultures of *Escherichia coli* after treatment with bacteriophage (Anderson, 1944, Luria and Delbruck, 1943, Luria, 1945) or X-ray (Tatum, 1945, Gray and Tatum, 1944) have demonstrated a variety of deficiencies in their metabolic processes Similar observations have been made on mutants induced in *Neurospora* by X-ray (Beadle, 1945)

Emerson (1944) has described a mutant of *Neurospora* which required sulfanilamide for growth and for which *para*-aminobenzoic acid was toxic This variant appeared in his cultures only once [8] The type B variants of meningococcus, on the other hand, developed regularly from all of 18 strains which included types I, II, and II alpha, some of which strains had recently been isolated from cases of epidemic meningitis and some from carriers, but others were old stock strains which had been under cultivation in the laboratory for many years

SUMMARY

Two streptomycin-resistant variants developed from each of 18 strains of meningococcus, including types I, II, and II alpha, when heavy seedings were planted onto a series of plates containing streptomycin in concentrations varying from 40 to 10,000 μg per ml One variant, designated type A, appeared in small and approximately equal numbers on all concentrations It grew in large yellowish colonies on streptomycin-free and streptomycin-containing media It retained the original virulence for mice possessed by its parent strain

The other variant, designated type B, appeared in greatest numbers on concentrations between 100 and 400 μg per ml, the concentrations optimal for its

[8] Personal communication to authors

multiplication   Its colonies varied in size and color depending upon the con
centrations of streptomycin on which they developed   They were small and
gray on concentrations of less than 100 μg per ml and larger and slightly yellowish
on concentrations of 200 μg or more per ml   Nevertheless, the type B variants
from any strain were found to be genetically identical and the differences in
their colonial appearance to be determined by the concentration of streptomycin
on which they grew

The type B variants were dependent on streptomycin for multiplication
in vitro and *in vivo*   They were nonviable on media containing concentrations
of less than 5 μg per ml and grew best on 100 to 400 μg per ml   They were
nonvirulent for mice, unless the mice received streptomycin   In mice treated
with streptomycin, they produced a fatal meningococcal sepsis and were re-
covered from the hearts' blood provided the cultures were made on streptomycin
containing media

Both variants retained the characteristic sugar fermentations of meningococci
and the type specificity of the parent strains from which they arose   Both
variants are presumed to arise from mutants which are constantly appearing
in the bacterial population of the parent strain

Whether the substance required by the B variants for their multiplication is
streptomycin itself or some impurity has not yet been determined   These
variants developed on all of 25 preparations of streptomycin obtained from 7
manufacturers   They failed to develop on streptomycin inactivated by hy
droxylamine or by cysteine

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ISOLATION AND CYTOLOGICAL STUDY OF A
FREE-LIVING SPIROCHETE

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The genus *Spirochaeta* includes the large, free-living, spiral microorganisms of the type described by Ehrenberg (1833) as *Spirochaeta plicatilis*   Zuelzer's (1910, 1912) careful studies of *Spirochaeta plicatilis* provide the most detailed description of the type species of the genus *Spirochaeta*   Zuelzer found this spirochete growing in both fresh- and salt-water enrichment cultures in close association with *Beggiatoa, Oscillatoria*, and other microorganisms   She described it as a flexible, spiral, blunt-ended organism 100 to 200, rarely 500, microns in length, $\frac{1}{2}$ to $\frac{3}{4}$ microns in diameter, and with regular, steep spirals having a wave length of 2 microns   The cells contained volutin granules   Motility was by screwlike, vibrating, flexing movements and, on solid surfaces, by a creeping movement   Multiplication was by simple or multiple transverse division   Zuelzer did not recognize a definite cell membrane, but some other early workers, as reviewed by Bosanquet (1911), described a definite "periplastic sheath"   Zuelzer considered the most distinguishing characteristic of *Spirochaeta plicatilis* to be a straight, elastic, axial filament around which the protoplasm was wound and which she observed in both living and stained cells

Other members of this genus have been described by Cantacuzene (1910), Dobell (1912), Zuelzer (1912, 1923), Pettit (1928), Gardner (1930, 1932), and other investigators   Unfortunately, all of the descriptions are based on preparations made from transient, mixed cultures, for no member of the family *Spirochaetaceae*, which includes *Spirochaeta, Saprospira*, and *Cristispira* (Bergey, 1947), has ever been reported grown in pure culture   Therefore, it has been impossible to make repeated and controlled studies on a given species, and, as a result, considerable confusion exists concerning the relationships of these microorganisms

We have succeeded in isolating a species of free-living *Spirochaeta*, believed to be *Spirochaeta plicatilis*, and have maintained it in pure culture for almost three years   This paper deals with the isolation and with the cultural characteristics and cytology of this spirochete

ISOLATION

Enrichment cultures of the spirochete were obtained from infusions of decaying leaves from a hydrogen sulfide spring   It was found that spirochetes, in association with *Oscillatoria, Beggiatoa*, and many other species of algae, bacteria, and protozoa from the enrichment cultures, grew sparsely on the surface of dilute leaf decoction agar plates   Microscopic observation of the plates revealed the

large, spiral, flexible spirochetes among the other microorganisms   Occasionally a group of spirochetes grew away from most of the other growth on a plate Such areas were marked and a speck of agar was carefully transferred to another plate   Repeated transfers on the decayed leaf medium eliminated many, but never all, of the contaminating species which were apparently contributing necessary growth factors   Addition of a few drops of blood to the leaf agar enabled the spirochetes to outgrow the last remaining contaminants   The pure culture of spirochetes obtained in this way was unable to grow on the leaf agar but grew well on the surface of medium containing 5 to 10 per cent sterile red blood cells and 1 to 1 5 per cent agar

Although only one strain has been isolated, spirochetes very similar in general appearance have frequently been observed in enrichment cultures from various ponds and springs in both Washington and New York states

CULTURAL CHARACTERISTICS

The spirochetes grow poorly or not at all on the serum fraction of blood but multiply readily on the red cell fraction, even when the red cells have been repeatedly washed   They have not been cultivated on any ordinary laboratory media, in liquid media, or on heat-sterilized blood   Hydrogen sulfide, although present in the spring from which the spirochetes were isolated, is apparently unnecessary for growth   The spirochetes are aerobic, grow in a pH range of 6 to 9 and in a temperature range of 15 to 34 C, the optimum temperature is about 26 C   Stock cultures are more successfully kept in small flasks than on slants and are transferred by washing off the growth with sterile water   Cultures remain viable for about a month at room temperature but not when stored for a similar period of time at refrigerator temperature

The spirochetes ordinarily grow slowly on the surface of the medium as a thin, spreading film just visible to the eye   Growth is apparent after one to several days   Examination of a plate culture with the low powers of the micro scope reveals the cells scattered on the surface as shown in figure 1 or often lying side by side with their spirals closely fitted together   Groups of such closely associated cells may advance at the edges of the diffuse growth in flamelike projections as shown in figures 2 and 3   They may occasionally pile up into discrete colonies as shown in figures 4 and 5, but this behavior is very uncommon

Motility on an agar surface is accomplished by a slow, forward, screwlike rotation of the cells, and sometimes a trail on the agar can be seen behind a moving spirochete like the track behind a snail   The motion of cells suspended in water is both screwlike and slowly but constantly flexing, and on moist surface one end of a cell may remain attached while the other back and forth   Cells may congregate in a droplet of moisture on a plate revolve around and around in it   Such a coiled cell, removed by making a cov slip impression, is shown in figure 12

MORPHOLOGY AND CYTOLOGY

The spirochetes have been studied at different ages by means of light-dark-field observation of living cells and by various staining procedures
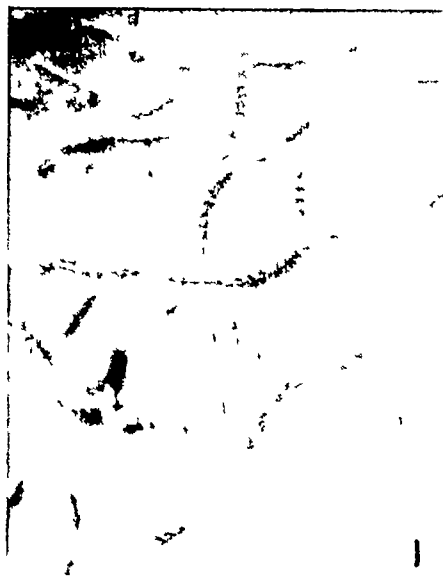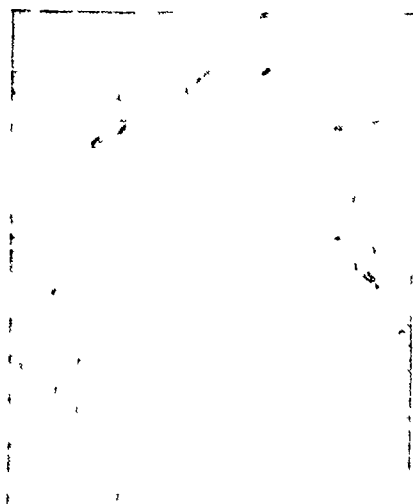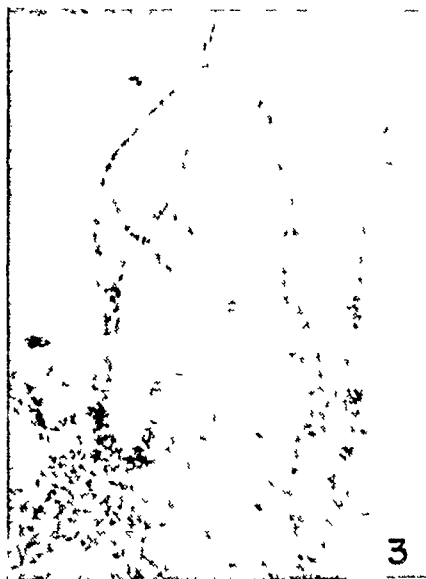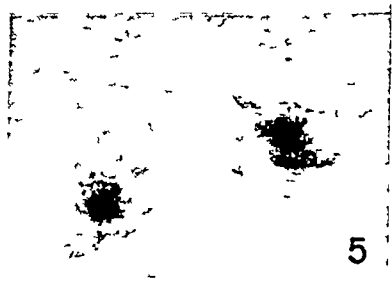
FIGS 1 to 5 Spirochetes growing on the surface of blood agar, photographed with side illumination
FIG 1 Typical growth with irregularly scattered cells   × 250
FIGS 2 AND 3 Groups of cells at edges of diffuse growth   × 250
FIGS 4 AND 5 Cells aggregated into colonies, this type of growth is not common   × 50

FIG 6 Living spirochetes photographed with the bright contrast phase microscope
(American Optical Company)   × 1,800

of the American Optical Company for allowing her to examine the spirochetes with the phase microscope and for taking the bright contrast phase photomicrograph of living cells which is shown in figure 6

FIGS 7, 8, AND 9 Spirochetes photographed with the 50 kv RCA electron microscope Most of the granules are volutin The cell shown in figure 9 has fragmented ✕ 18 000

The living spirochetes are flexible, regular spirals with a diameter of 0 8 to 1 2 microns and a length ranging from 1 to 2 wave lengths to several hundred microns Individuals 400 microns long are fairly common, and even longer ones

occui occasionally   That the spiiochetes are spiral instead of wavy can be seen
by focusing and is appaient on the phase photomicrogiaph   In young spi
rochetes the wave length vaiies fiom 3 to 6 5 microns depending on how tightly

10   **11**
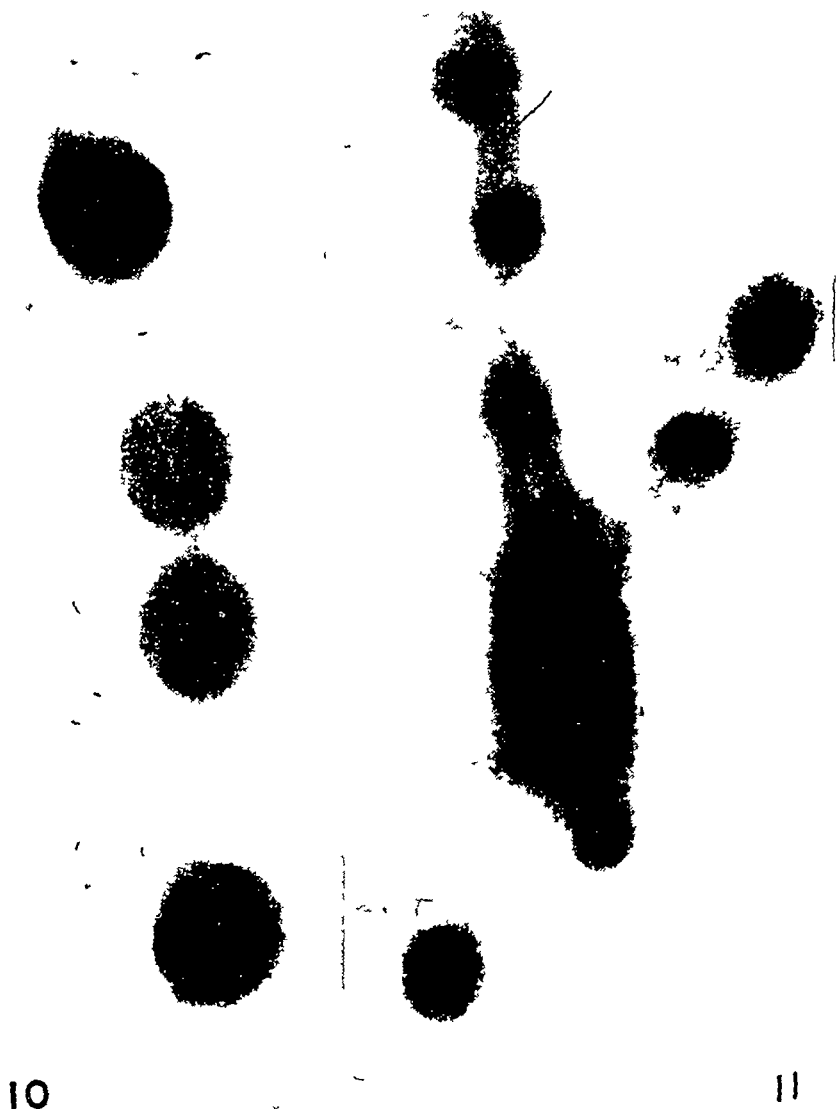
FIGS 10 AND 11 Spirochetes photographed with the 50 kv RCA electron microscope
× 36,000

the spiral is coiled, and the spiial amplitude is about 2 microns   However,
these dimensions become more inconstant in old cells, which may either unwind
into loose, irregulai spirals or straighten out almost completely   Likewise,
cells dried and fixed usually become straight
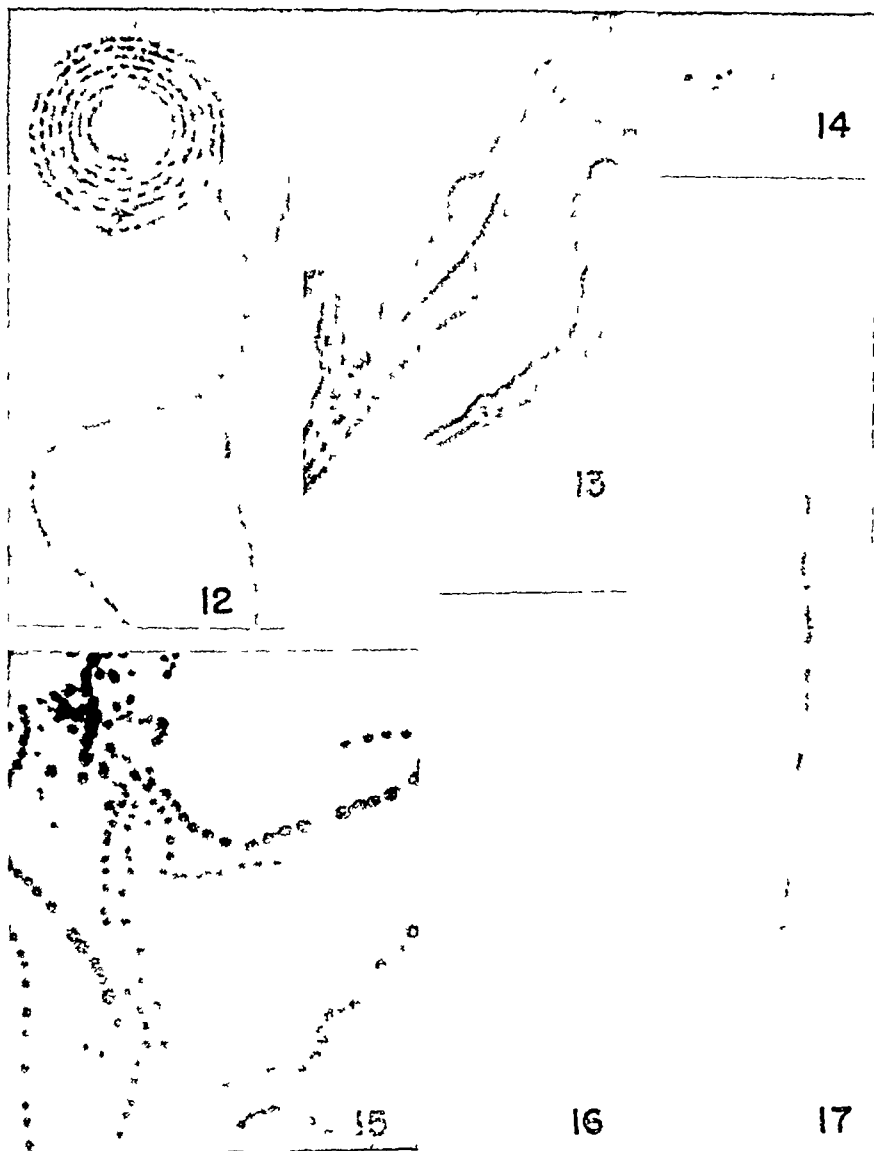
FIG 12 Coiled spirochete removed from a plate by making a cover slip impression, fixed and stained with Giemsa's solution   × 1,350

FIG 13 Spirochetes stained with a cell wall stain   One cell has retained the spiral form and another shows the cell membrane distended by volutin granules   × 2 250

FIG 14 Spirochete from an old culture showing a swelling at one end   × 1 350

FIG 15 Spirochetes stained with methylene blue to demonstrate volutin inclusion × 2,250

FIG 16 Cell of a large *Spirillum* stained with a flagella stain to illustrate the appearance of an 'axial filament "   × 2 500

FIG 17 Spirochete cells stained with Giemsa's solution to demonstrate the axial filament "   × 2,500

In old cultures the cells sometimes form swellings, as in figure 14, similar to the plasmolysis figures reported by Dobell (1912) for *Saprospira flexuosa* Such cells may still be motile

Many refractive granules are apparent in spirochetes examined by either light- or dark-field illumination The inclusions are of at least two types volu tin, which has frequently been reported to occur in free-living spirochetes, and fat We have followed the inclusions at intervals in cultures from several days to more than a month old The volutin inclusions are identified by their intense and metachromatic staining with methylene blue and by their solubility in hot water and in 0 02 per cent NaHCO₃ Volutin granules in cells stained with methylene blue show clearly in figure 15 These inclusions are arranged either regularly or irregularly in the cell and range in size from small specks to relatively immense bodies occupying the entire diameter of the cell The larger ones are often compressed into a rectangular shape and may stain more intensely around their border with basic dyes, suggesting a vacuolar rather than granular char acter They are present in greatest abundance and size in cultures a few days to a week old but persist in many cells even in month-old cultures Most of the inclusions seen in the electron photomicrographs are volutin Granules of a volutin nature frequently persist after old cells have disintegrated, there is no evidence that these represent anything but degenerated cells

The fat inclusions are identified by deep staining with Sudan black B when cells are suspended in a saturated ethylene glycol solution of this dye Cells 4 to 7 days old contain many fat granules, and some cells from month-old cul tures still contain small ones The volutin and fat inclusions are distinct from each other, as may be observed when Sudan black B solution is allowed to run under a cover slip onto a film already stained with methylene blue

The electron photomicrographs show the presence also of inclusions too minute to be resolved by the light microscope The cytoplasm stains with basic dyes and is gram-negative

Spirochetes of various ages have been hydrolyzed with N HCl at 60 C for 10 minutes and stained both by the Feulgen method and with Giemsa's solution By either method the cells appear somewhat granulated or stippled, this effect being more apparent when they are examined wet than when in oil A few Feulgen-positive granules were observed which may be nuclei, but more work should be done before this is certain

A cell membrane, which does not stain readily with Giemsa's solution or with basic dyes, may be demonstrated with Dyar's (1947) cell wall stain The mem brane in the living cell is, of course, flexible and in a fixed, stained cell shrinks in close to the cytoplasm even when examined wet The cell wall stain shown in figure 13 shows the membrane shrunk except where it is distended by large volutin granules, presenting much the appearance of a tight rubber skin stretched over a string of beads Delicate, refractive cross walls have been seen in a few living cells examined by dark-field illumination, and, likewise, occasional in dividuals when stained by the cell wall method are seen to consist of shorter cells, each with a complete membrane around it The spirochetes with pro

walls seen by these methods are relatively few and rather clearly represent a stage of multiple transverse division

Dried or fixed cells sometimes fragment into regular segments each about half a wave length long as shown in the electron photomicrograph in figure 9   However, the prominent, regular cross striations which are seen in specimens of *Sapiospira* and *Cristispira* stained with Giemsa's stain or with basic dyes and which give these organisms their characteristic "chambered" appearance are not evident in stained specimens of this spirochete nor in the electron photomicrographs   Nor are regular cross striations detectable in living cells by either ordinary light- or dark-field illumination

However, examination with the phase microscope (Bennett, Jupnik, Osterberg, and Richards, 1946) reveals fine, delicate cross septae occurring throughout the length of living cells   In young cells the septic are very striking for their clarity and regular spacing at half turns of the spiral in all cells   In old cells the cross walls are also clearly present, although they may be somewhat less distinct and more irregular in spacing   The spirochetes are clearly not single, long spiral cells but multicellular spiral filaments

No flagella have been demonstrated with Leifson's (1930) flagella stain, nor do the electron photomicrographs give any evidence of flagella

An investigation of the "axial filament" seemed especially important because of the prominence it has been given in characterizing *Spirochaeta*   Zuelzer (1910) was the first to describe this structure as a straight, elastic filament around which the protoplasm was wound   Some other investigators such as Bach (1921) and Gardner (1930) have confirmed its presence   On the other hand, Dobell (1912), although he accepted the concept of an axial filament, was unable to observe it in the several species of *Spirochaeta* that he studied, and Noguchi (1928) was unable to demonstrate it in a spirochete from the slime of an icebox drain   Certainly not every investigator will admit its existence

In this spirochete, an "axial filament" has been demonstrated, as shown in figure 17, but only rarely in preparations stained overnight in Giemsa's solution and by no other method   It has been apparent only in preparations where the cells are heavily outlined with stain as a result of the long staining time, and then only in cells that have dried in the spiral form, never in straight individuals even though they be adjacent on the slide   The "axial filament" appears to be continuous with the heavily stained cell outline   The outline ordinarily is apparent just at the cell borders where one is looking through the greatest thickness of stain   However, in a spiral cell the places where the cell spirals around also present a greater thickness, resulting in the appearance of a heavily stained filament lying in the axis of the spiral   Giemsa staining does not show such a structure in spiral cells of *Spirillum*, probably because it is masked by the entire cell's being very intensely stained, but, indeed, the appearance of an axial filament can be produced in a large species of *Spirillum*, as shown in figure 16, by the use of Leifson's flagella stain, a procedure which precipitates stain on the cell   In this *Spirillum* no such structure is apparent by any other procedure

Therefore, an axial filament is thought to be an artifact resulting from the appearance of stain deeply outlining the spiral form   The light red cell wall stain does not seem to be intense enough to duplicate this effect

Neither examination of living spirochetes with the phase microscope nor the electron photomicrographs show any evidence of an axial filament in this spirochete   Likewise, electron photomicrographs of *Treponema pallidum* taken by Morton and Anderson (1942), of three species of *Treponema* taken by Mudd, Polevitzky, and Anderson (1943), and of *Borrelia novyi* taken by Lofgren and Soule (1945) show no axial filament in these organisms

<div align="center">DISCUSSION</div>

It is apparent that there is a very close resemblance between this *Spirochaeta* and *Spirochaeta plicatilis* in regard to natural habitat, cell form and size, type of cell inclusions, division, and motility   The dimensions do not coincide exactly with those reported by Zuelzer for *Spirochaeta plicatilis*, however, we do not believe that the inconsistency represents a real difference, because our cells were measured alive and hers presumably were measured after fixing and staining   Actually, a stained cell such as the spiral one in figure 13 of this paper and the ones shown in table 1 in Zuelzer's (1912) paper are almost identical in respect to diameter, wave length, and spiral amplitude   Furthermore, although we consider the "axial filament" as an artifact, the appearance of such a structure has been obtained with Giemsa's solution, one of the procedures that Zuelzer used to demonstrate it

There seems little question that the free-living *Spirochaeta* which we have isolated is identical with the type species, *Spirochaeta plicatilis*

The presence of an axial filament and the absence of a distinct periplast membrane and of prominent cross striations in stained specimens are three important characteristics used to differentiate *Spirochaeta* from *Saprospira* and *Cristispira* (Bergey, 1947), *Cristispira* is further separated on the basis of its crista and parasitic habitat   However, these criteria surely need reconsideration because in the present study we have thrown considerable doubt on the reality of the axial filament   Furthermore, although the membrane and cross septae of this spirochete are not readily apparent by usual procedures, a membrane is clearly demonstrable by a cell wall stain, and regular cross septae by observation of living cells with the phase microscope   We are not convinced, on the basis of the present knowledge, that *Spirochaeta* and *Saprospira*, as described by Gross (1911), should be considered distinct genera

We believe that a truly satisfactory relationship among the *Spirochaetaceae* can be established only when more representatives of this group have been isolated and studied under reproducible and comparable conditions

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milk, one was from the diseased lung of a dog, one, received from the American Type Culture Collection, had a history of having been isolated from pus from a horse with strangles   The authenticity of this origin is questionable, because the strain came indirectly from the original investigator and was labeled "S equi "

Some of the strains listed in table 1 are duplicates from the same patient It appears that more than one of the strains (nos 1188, 1308, 1355, and 1357) that were received from Dr Sherman may have been isolated at different times from the same patient, because, according to Sherman, Stark, and Mauer (1937), S zymogenes was isolated on various occasions from one subject Strains 1332 and 1333 were originally the same strain, one of the branches having had a history of undergoing variation in its ability to hemolyze blood

THE CHARACTERISTICS OF THE ENTEROCOCCI

The strains included in table 1 belonged to group D, according to Lance field's precipitin test, all grew at 10 C and 45 C, all grew in media containing 6 5 per cent NaCl and in media having an initial pH value of 9 6, all tested strains (31) grew in media containing 40 per cent bile, all hydrolyzed esculin, none hydrolyzed starch, all attained a final pH of 4 4 or lower in glucose broth, except one (no 945) which produced a final pH of 4 6, all strains fermented maltose, of 27 strains tested, all fermented salicin, of 29 strains tested, all fermented trehalose   None of 25 tested strains fermented dulcitol or inulin,

The characteristics which were common to all strains are omitted from table 2, which includes those reactions which showed interesting differences between strains   Omitted from the table are the following reactions hydrolysis of sodium hippurate, production of ammonia in 4 per cent peptone, and virulence for mice These reactions appeared not to be correlated with significant characteristics

Though not considered in table 2, a general statement in regard to the viru lence of the enterococci for mice may be of interest   Mice were injected intra peritoneally with broth cultures, which had been inoculated very lightly by platinum needle and incubated for about 11 hours   Two strains killed mice in 10^{-2} dilution, 22 killed in 10^{-1} dilution but not in higher dilutions, 9 failed to kill in the 10^{-1} dilution   According to these results, the virulence enterococci for mice is low as compared with the virulence of many strains streptococci of groups A and C

Uncorrelated with characteristics which appeared to be significant for fication purposes are the following, listed in table 2 type of hemolysis, liquir tion of gelatin, sensitivity to bacteriophage D_2-1188, production of acid lactose, arabinose, and raffinose   The characteristics of distinction were to be agglutinative reactions, sensitivity to phage D-693, growth in milk taining 0 1 per cent methylene blue, survival at 60 C for 30 minutes, duction of acid from sucrose, mannitol, and sorbitol   In the study of a series of strains, the production of acid from glycerol might be found to some significance

TABLE 1

Histories of streptococcal strains of group D

NIH NO	PREVIOUS INVESTIGATORS	PREVIOUS DESIGNATION	SOURCE		
			Host	Material	Disease
693	Kendrick and Hollon, Abbott Laboratories	*S fecalis* no 31 1900 89	Human	Feces	Intestinal hemorrhages
696	The Lilly Research Laboratories	*S hemolyticus* no 1527	Human	Pus	Empyema
702	Allen Sandlin Laboratories		Human		Sore throat
894	Graham	18297	Canine	Lung	
912	Thompson and Megrail	S 56	Human	Tooth	Infected
913		S 112	Human	Feces	None
914		S 115	Human	Feces	None
945	Dr John S Buckley, Am Type Culture Collection, *Catalogue of Cultures*, 1927	A M S 6, 827	Equine?	Pus?	Strangles?*
977	Torrey and Montu	Intestinal streptococcus no 11	Human	Feces	Chronic ulcerative colitis
978		Intestinal streptococcus no 13	Human	Lesion	Chronic ulcerative colitis
979		Intestinal streptococcus no 24	Human	Feces	Chronic ulcerative colitis
980		Intestinal streptococcus no 14	Human	Feces	None
1121	Lederle Laboratories	L-14	Human		Osteomyelitis
1122		L-50	Human	?	?
1130		32	Human	Pus	Peritoneal abscess
1131		198	Human	Heart blood	Septicemia following smallpox vaccination
1132		291	Human	Pus	Ear infection following measles
1181		Hilligest	Human		Meningitis following mastoiditis
1187	Farrell	R-36	Human		Normal throat
1188	Sherman, Stark, and Mauer		Human	Feces	Diarrhea
1275	Saunders, Torrey and Montu	66	Human		Gastric ulcer carcinoma
1276		83	Human		Gastric ulcer
1278		140	Human		Gastric ulcer carcinoma

TABLE 1—*Continued*

NIH NO	PREVIOUS INVESTIGATORS	PREVIOUS DESIGNATION	SOURCE		
			Host	Material	Disease
1308	Sherman, Stark, and Mauer	*S zymogenes* no 1	Human	Feces	Intestinal disorder
1309	Sherman and Wing	*S durans* Wing 3		Milk powder	
1332	} Dr E C Rosenow	257 21†	} Human		} Endocarditis
1333		257 18†			
1355		26C$_1$	Human		
1357	} Sherman, Stark, and Mauer	36C$_2$	Human		} Intestinal disorder
1359		132B		Pasteurized milk	
1531		Varn	Human		Peritonitis
1574		Rigens	Human	Pus	Otitis media
1588		3365	Human	Pooled plasma	
1600		31A	Human	Pooled plasma	

* The authenticity of the source of strain 945 is questioned (see the text)
† Strains 1332 and 1333 were from the same patient

TESTS FOR THE DIFFERENTIATION OF ENTEROCOCCAL SPECIES

According to the key in the fifth edition of *Bergey's Manual* (1939) liquefac tion of gelatin and hemolysis are considered to be distinctive characters, on which the differentiation of enterococcal species is based   Sherman, Stark, and Mauer (1937) mentioned the "thin and shaky boundaries" which separate the "supposed" enterococcal species, but Sherman (1938) recognized 3 species which he differentiated on the basis of the two characteristics mentioned above

Durand and Dufourt (1923) reported that they found a precise correlation between liquefaction of gelatin and agglutinative reactions   According † other investigators, however, liquefaction of gelatin is an unstable property · no significance in classification   Houston (1934) noted that the action bacteriophage may alter the gelatin-liquefying property   Elser and Th' (1936) found gelatin-liquefying strains of enterococci which agreed well nonliquefying strains in cultural and biochemical properties   Wheeler Foley (1943) stated that biologic characteristics of enterococci could not correlated with serologic type

Lack of correlation between liquefaction of gelatin and significant acteristics may be noted in table 2   For example, strains 1278 and 1600 almost identical reactions in all tests excepting that for liquefaction of b

Gordon (1922) found that hemolytic and nonhemolytic strains of ente behaved alike in agglutinin absorption tests   Frobisher and Denny Elser and Thomas (1936), and Sherman and Stark (1931) found that h and nonhemolytic strains resembled each other in every respect exc ʲ reaction on blood   The literature records many instances of the loss ·

Characteristics of the enterococci

NO	AGGLUTINATION IN SERUM			SENSITIVITY TO FILTERED PHAGE		BETA HEMOLYSIS	LIQUEFACTION OF GELATIN	METHYLENE BLUE*	SURVIVAL 60 C, 30 MIN	ACID PRODUCTION FROM						
	1188	1130	894	D/693	D7/1188					Arabinose	Sucrose	Lactose	Raffinose	Mannitol	Glycerol	Sorbitol
Streptococcus zymogenes (MacCallum and Hastings)																
1188	500†	100	1,000	+	+	+	−	+	+	−	+	+	−	+	+	+
1308	500	100	1,000	+	+	+	−	+	+	−	+	+	−	+	+	+
914	500	100	1,000	+	+	+	−	+	+	−	+	+	−	+	+	+
1275	500	1,000	1,000	+	+	−	−	+	+	−	+	+	−	+	+	+
1276	500	1,000	1,000	+	+	−	−	+	+	−	+	+	−	+	−	+
1278	500	1,000	1,000	+	+	−	−	+	+	−	+	+	−	+	−	+
1600	100	100	500	+	+	−	+	+	+	−	+	+	−	+	−	+
1355	10	100	10	+	+	+	+	+	+	−	+	+	−	+	+	+
693	100	50	−	+	+	+	−	+	+	−	+	+	−	+	+	+
702	500	100	1,000	+	−	+	−	+	+	+	+	+	+	+	+	+
912	500	1,000	+	+	−	+	−	+	+	−	+	+	−	+	+	+
1357	500	−	1,000	+	−	+	−	+	+	−	+	+	−	+	+	+
1588	100	−	100	−	−	−	+	+	+	−	+	+	−	+	−	+
1359	500	100	−	+	−	+	−	+	+	−	+	+	−	+	+	+
980	100	10	100	+	−	+	+	+	+	−	+	+	−	+	+	+
1531	100	100	500	+	−	−	−	+	+	−	+	+	−	+	+	+
913	100	100	10	+	−	+	+	+	+	−	+	+	−	+	+	+
Group 2																
1130	−	10	100	+	−	+	−	+	+	−	−	+	−	+	+	−
1122	−	10	100	+	−	+	−	+	+	−	−	+	−	+	+	−
1121	10	50	10	+	−	+	−	⊤	+	−	−	+	−	+	+	−
1131	−	10	−	+	−	+	−	+	+	−	−	+	−	+	+	−
977	10	10	−	+	−	+	+	+	+	+	−	+	−	+	+	+
978	−	1,000	−	−	−	+	−	+	+	−	−	+	−	+	+	+
1187	−	100	−	+	−	−	−	+	+	+	−	+	−	+	+	+
696	−	100	1,000	+	−	+	−	−	+	−	+	−	−	+	+	+
Group 3																
1132	−	−	100	+	−	+	−	+	+	−	−	+	−	+	+	−
979	−	−	100	+	−	+	−	+	+	−	−	+	−	+	+	+
894	−	−	1,000	+	−	+	−	+	+	−	−	+	−	+	+	+
945‡	−	−	10	-		−	−	+	+	+	+	+		+	−	+
Group 4																
1181	−	−	−	+	+	+	−	+	+	−	+	+	−	+	+	+
1574	−	−	−	+	+	−	+	+	+	−	+	+	−	+	+	+
Group 5																
1332	−	−	−	−	−	−	−	+	−	+	+	+	+	+	−	+
1333	−	−	−	−	−	−	−	+	−	+	+	+	+	+	−	+
S. durans (Sherman and Wing)																
1309	−	−	−	−	−	+	−	−	+	+	−	+	−	−	−	−

* Strong reduction in milk containing 0 1 per cent methylene blue

† The titers are expressed as reciprocals of the highest serum dilution which agglutinated

‡ Strain 945 was peculiar in that the final pH was only 4 6

lytic power in streptococci (Grinnell, 1928, Todd, 1928, Fry, 1933, Lancefield, 1934)   That this phenomenon may occur in enterococci was observed by several investigators (Gordon, 1922, Stein, 1933, Noel, 1934)   Our strain 1333 was originally a hemolytic strain which, when it was received, had a history of having produced a green variant from a pure line culture   In our laboratory neither strain 1333 nor the substrain 1332 produced beta hemolysis   The reverse change, the acquisition of hemolytic power by enterococci of the alpha type, was reported by Meyer (1926)

In our laboratory repeated changes in type of hemolysis were observed in strain 693   This strain had been isolated by Kendrick and Hollon (1931) from feces in a case of intestinal hemorrhages   They noted that when first isolated it was strongly hemolytic, but that it soon lost its hemolytic power and became alpha hemolytic   After transmission to our laboratory, however, it produced beta hemolysis   Because it did not behave in accordance with its previous history, another subculture of the strain was requested   It also produced beta hemolysis in our laboratory when first received, and it has done so consistently   However, a subculture of our beta hemolytic 693 was given to another laboratory of the National Institute of Health, and it was reported as having changed to an alpha hemolytic strain   A subculture of this alpha strain was returned to our laboratory   It was tested for type of hemolysis on agar containing rabbit blood, which was in general use in our laboratory, and on sheep blood, which was in use in the other laboratory   On rabbit blood agar, beta hemolysis occurred, but on sheep blood, alpha hemolysis occurred   This observation of differences in the type of hemolysis dependent on the source of blood was made also by Kobayashi (1940), who reported that the enterococcus does not hemolyze the blood corpuscles of the goat or sheep, although it may hemolyze the corpuscles of man, horse, cow, or rabbit

The inconstancy of hemolysis in strain 693 illustrates the unreliability of the hemolytic property as a character of specific significance   The lack of correlation between type of hemolysis and other characteristics is illustrated in table 2 by strains 914 and 1275, which gave almost identical reactions in all tests excepting that for hemolysis

Enterococcal bacteriophage   That enterococcal bacteriophage may be more widespread than phages attacking other streptococci is suggested by the more frequent mention of it in the literature   Beckerich and Hauduroy (1922), and also Hadley and Dabney (1926), studied this phage   Bagger (1926) reported the sudden appearance of bacteriophage in a plate culture of a strain of enterococcus which had been under cultivation for a long time, many similar plate cultures having been made previously   According to Houston (1936), an active enterococcal phage can often be isolated from the stools in cases of ulcerative colitis   This author also reported (1934) that in a septic focus the enterococcus usually occurs in a phage-infected form   He believes that the action of the phage results in variation in the characteristics of the organism   Graham and Bartley (1939) found that 34 of 36 strains of enterococci were sensitive to all three phages which they studied   In our experience, enterococcal phage could be readily obtained from sewage

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ε and Hollon (1931) noted the parallelism between serologic and bacteric relationships in a group of fecal streptococci One of us (A C E) their phage, and reported (1934) that sensitivity to this phage, designated differentiated the enterococci from other streptococci, and that on the bacteriophagic reactions the grouping of enterococci corresponded with grouping recognized on the basis of other characteristics

ng the course of our studies on bacteriophage, a race designated D_2 was n a sample of sewage taken in Washington, D C It lysed enterococci, tered from phage D in that the antilysins prepared against the two phages d differently Antilysin D_2 neutralized phage D as well as phage D_2 as antilysin D neutralized the homologous phage but not phage D_2

...ch phage was prepared by propagation on a strain of enterococcus found to be highly sensitive Phage D was propagated on strain 693, and phage D_2 was propagated on strain 1188 The techniques of isolating the phage, preparing the filtrates and the antilysins, making the serologic tests, and determining the sensitivity of the streptococci to the phages were described in previous publications (Evans, 1934, 1942, Evans and Socknder, 1942) All strains of enterococci were tested for sensitivity to filtrates of both phages, D and D_2, with results as shown in table 2

Serologic relationships A number of investigators (Gordon, 1922, Durand and Dufourt, 1923, Meyer and Lowenstein, 1926, Takeda, 1935, Meyer, 1937) reported that the majority of enterococci from various sources fall into a few well-defined groups according to agglutinative reactions, which were confirmed by agglutinin absorption tests in the studies of some of the investigators

Saunders (1930) reported that in a large series of cases enterococci from the tissues of resected gastric and intestinal ulcers and from certain types of ulcers in other parts of the body exhibited similar serologic characteristics Torrey and Montu (1936) found that enterococci serologically related to Saunders' strains occurred more frequently in patients showing intestinal lesions than in normal adults A few of Saunders' strains (nos 1275, 1276, and 1277 of tables 1 and 2) and a few of Torrey and Montu's strains (nos 977, 978, 979, and 980) were available for the present study It was found that the strains from cases of ulcer, received from Saunders and from Torrey and Montu, resembled strains from other pathologic as well as nonpathologic sources in serologic behavior as well as in physiologic and biochemic reactions

That the commonest serologic types of enterococci are widely distributed is suggested by the studies of the following authors Houston (1936) reported that type 1 of the "Belfast classification" was identical with one of Meyer's types of continental European strains, and Meyer (1937) reported that his type 1 agreed with Takeda's (1935) type 1 of strains isolated in Japan

Agglutinating serums were prepared against three strains, 894, 1130, and 1188, strain 1188 having been selected because it was the strain previously utilized for the propagation of phage D_2 Strain 1130 was selected to represent strains which failed to agglutinate in antiserum 1188, strain 894 was selected to represent strains which failed to agglutinate in either serum

Rabbits were injected with antigen prepared from fresh broth culture heated at 56 C for 1 hour   The cultures were centrifuged and the sediments were suspended in saline solution containing 0 2 per cent tricresol   The first 3 doses, injected subcutaneously on successive days, each consisted of 0 5 ml of antigen of a turbidity equivalent to 2,000 ppm of the silica standard   They were followed by 2 intraperitoneal doses of 1 ml, 400 ppm, on successive days   Intra peritoneal injections were made with increasing doses 3, 4, or 5 times during the following 2 weeks, the largest being 1 0 ml, 1,000 ppm   Trial bleedings were then made 6 days after the last injection   If the serum did not show a good titer of agglutinins, further injections were made

In the case of strain 1188, a satisfactory serum was obtained after treatment of the rabbit for 3 weeks, strain 894 required 4 weeks   In the case of strain 1130, the serum was unsatisfactory, when tested against the homologous strain, after treatment for 6 weeks   It agglutinated in 1 10 but not in higher dilutions However, it agglutinated many heterologous strains in a titer of 1 100 or 1 1,000 (table 2)   Torrey and Montu (1936) reported that some of their strains lacked agglutinogenic properties

Distinguishing characteristics of the enterococci   In table 2 the 34 strains of enterococci are arranged in 6 groups, primarily according to agglutinative reactions   It may be noted that certain other characteristics are more or less correlated with agglutinative reactions

The first group includes 17 strains, one of which, no 1308, was labeled *Streptococcus zymogenes* when received from Dr Sherman   All strains of this group agglutinated in serum 1188, all but two agglutinated in serum 1130, and all but another two agglutinated in serum 894   All strains were sensitive to phage D-693, all grew in milk containing 0 1 per cent methylene blue, all survived 60 C for 30 minutes, all produced acid from sucrose, lactose, mannitol, and sorbitol   The strains varied in sensitivity to phage D_2-1188, hemolysis, liquefaction of gelatin, and production of acid from glycerol   Only one strain produced acid from arabinose, and it was also the only one which produced acid from raffinose   The table shows that liquefaction of gelatin and type of hemolysis are uncorrelated with other characteristics   According to all available evidence the 17 strains of the group belong to one species   In agreement with priority of nomenclature the group should be designated *Streptococcus zymogenes* (Mac Callum and Hastings, 1899), and the names *faecalis* and *liquefaciens* should be eliminated

The last group in table 2 includes only one strain, no 1309, which was received from Dr Sherman with the designation "*S thermodurans*"   Afterwards Sherman and Wing (1937) changed the specific name to *Streptococcus durans* These authors reported that *S durans* does not have as strong a reducing action as other strains of group D, it lacks the ability to produce acid from glycerol and sorbitol, usually it does not attack mannitol or sucrose   These distinguishing characteristics of *S durans* were confirmed in our studies (table 2), which show, further, that strain 1309 does not agglutinate in the serums which

agglutinate the strains of *S zymogenes*, and that it is resistant to phages D-693 and D₂-1188

Between the widely different species *S zymogenes* and *S durans* are strains of intermediate characteristics (see table 2) These intervening groups show a gradation of divergence from the characteristics of *S zymogenes* toward the characteristics of *S durans* Group 2 differs from *S zymogenes* chiefly in the failure to agglutinate in antiserum 1188, and in the failure of most of the strains to produce acid from sucrose and sorbitol Group 3 diverges further in that the strains fail to agglutinate in antiserum 1130 The strains of group 4 agglutinate in none of the serums, but in other characteristics they resemble those of *S zymogenes*

The data in table 2 are insufficient to determine whether any one or more of the groups 2, 3, and 4 or any combination of them should be regarded as a separate species However, the failure of production of acid from sucrose and sorbitol appears to have some significance Possibly a new species should be recognized largely on the basis of those characteristics

The strains of group 5, one of which was derived from the other, are clearly differentiated from the two recognized species, *S zymogenes* and *S durans* They differ from *S zymogenes* in their failure to react in the three agglutinating serums, in their resistance to phage D-693, and in their failure to survive 60 C for 30 minutes They differ from *S durans* in their strong reduction of methylene blue, in their failure to survive 60 C for 30 minutes, and in their production of acid from sucrose, mannitol, and sorbitol

Graham and Bartley (1939) mentioned a variety of enterococcus which lacked the property of resistance to heat Another author mentioned strains which resisted 60 C for 10 minutes, but not for 30 minutes This report was seen by one of us (A C E), but the reference was lost If group 5 is found to be of numerical significance, it should be given a specific name

Enterococci in various animal hosts The hardiness of the enterococci enables them to multiply under a wide variety of conditions They are found in health and disease, not only in various species of mammals, but also in lower forms of animal life Steinhaus (1941) isolated enterococci from 5 species of insects, Sherman (1937) quoted several authors who considered the so-called *Streptococcus apis*, which is associated with European foul brood of bees, to be an enterococcus Plummer (1941) isolated an enterococcus from the eye of a ferret, Sylvester and Benedict (1941) isolated it from the viscera of foxes and minks, and Elser and Thomas (1936) isolated it from the cervix of guinea pigs Orcutt (1926) found enterococci in the digestive tract of normal calves and in calves suffering from diarrhea or scours Torrey and Montu (1936), and also Plummer (1941), studied enterococci which had been isolated from milk in cases of bovine mastitis Two of our strains, nos 1309 and 1359, were probably of bovine origin, not associated with disease

Hont and Banks (1944) cultivated enterococci from a pig which died of endocarditis, and they produced disease in a young pig from a healthy herd by

that 73 out of 100 strains of streptococci isolated from specimens of urine belonged to group D   Hollander (1942) found that 22 out of 40 (55 per cent) of streptococci from infections of the genitourinary tract belonged to group D Rantz and Kirby (1943) found 27 per cent of streptococci isolated from specimens of urine belonged to group D   From 5 of their cases which presented signs of pyelonephritis they isolated enterococci in pure culture

Puerperal sepsis   A good many authors have reported finding enterococci associated with puerperal sepsis (Meyer and Lowenstein, 1936, Ehrismann, 1935)   Gordon (1922) found them in 8 cases, Ramsay and Gillespie (1941) in 6, and Rantz and Kirby (1943) in 2   Witebsky and his coworkers (1939) isolated a strain of enterococcus from the blood in a case of septicemia following abortion

Takezawa (1937) found 50 strains of enterococci among 216 strains of streptococci from female genital organs in various diseases   Hare and Colebrook (1934) isolated streptococci with the characteristics of enterococci from 7 of 34 women who had low-grade fever during the puerperium, but in only a few instances were the organisms isolated in pure culture   Lancefield and Hare (1935) found no streptococci of group D among 46 strains from cases of severe infection of the uterus, but they found 8 strains of group D among 18 strains from "minor infections "   Brown and Schaub (1945) found that 9 per cent of 232 strains of streptococci from the uteri of patients with febrile puerpera were enterococci

Otitis media, mastoiditis, and meningitis   Thiercelin (1899) isolated enterococci from cases of meningitis, and Andrewes and Horder (1906) found them in cases of otitis media, mastoiditis, and meningitis   Subsequent investigators have confirmed those early reports   Ehrismann (1935) quoted two authors who reported cases of meningitis due to enterococci, Lang, Lode, and Schuttermayer (1937) reported 2 cases, Wheeler and Foley (1943), 1 case   Rantz (1942) reported 1 case in which meningitis followed a prolonged ear infection   Rantz and Kirby (1943) found that about 10 per cent of streptococcal infections of the middle ear were caused by enterococci

Among the strains of our collection, no 1132 was from a case of ear infection following measles, no 1181 was from a case of meningitis following mastoiditis, and no 1574 was from a case of otitis media

Endocarditis   MacCallum and Hastings (1899) obtained an organism which they called *Micrococcus zymogenes* from the blood in a case of endocarditis, ' Sherman (1937) was convinced that their organism was an enterococcus   Subsequently many authors reported the isolation of enterococci in cases of endocarditis (Andrewes and Horder, 1906, Hicks, 1912, Gordon, 1922, Meyer Löwenstein, 1926, Dible, 1929, Wallach, 1934, Houston, 1934, Baum, 1. Ehrismann, 1935, Elser and Thomas, 1936, Reiners, 1936, Fox, 1936, W. 1937, Clements, 1937, Otto, 1938, Moran, 1938, Rohleder, 1938, Williams, . Lederle, 1940, Skinner and Edwards, 1942, Rantz and Kirby, 1943, Wheel Foley, 1943, MacNeal and Blevins, 1945, Brown and Schaub, 1945) strains of our collection, nos 1332 and 1333, were from one case of ma endocarditis

Some of the investigators mentioned reported on the frequency of occurrence of enterococci in their cases of endocarditis Elser and Thomas encountered enterococci "not infrequently" in the blood of patients suffering from a subacute form of endocarditis Andrewes and Horder found 4 strains of enterococci among the streptococci from 24 cases of malignant endocarditis, Dible reported that 1 strain out of 6 isolated from the blood in cases of ulcerative endocarditis was an enterococcus, Moran found it in 5 out of 20 cases, Lederle in 8 out of 10 cases, Rantz and Kirby in 3 out of 16 cases, and MacNeal and Blevins in 6 out of 36 cases

The portal of entry was determined in a number of cases of endocarditis reviewed by Skinner and Edwards (1942) Enterococci obtained from the blood stream were derived from an infected finger in 1 case, infected tonsils in 1, the gall bladder in 2, the urinary tract in 2, septic abortion in 3, and the gastrointestinal tract in 5

Miscellaneous diseases Rantz and Kirby (1943) called attention to the fact that the enterococci are rarely found associated with infections of the respiratory tract They reported 8 cases, Wheeler and Foley (1943) reported 1 case Brown and Schaub (1945) found enterococci in mixed cultures in cases of pneumonia One strain of our collection (no 702) was from a case of sore throat

The enterococcus is occasionally reported to be associated with various diseases not mentioned above Meyer and Lowenstein (1926) found it in cholecystitis, osteomyelitis, and pancreatitis, Houston (1934) found it in septic tonsils, the root canal of septic teeth, postnasal catarrh, septic antra, excised gall bladders, abscesses in various parts of the body, certain forms of acne and other skin lesions, and invariably in chronic onychia, Wheeler and Foley (1943) found it in dermatomyositis and in emphysema Our collection includes strains from empyema (no 696), an infected tooth (no 912), osteomyelitis (no 1121), and septicemia following smallpox vaccination (no 1131)

There is an extensive literature on the association of enterococci with rheumatic diseases It is omitted here because, if the association should be proved to be significant, this literature should be treated separately

SUMMARY

The literature on human infections with enterococci is reviewed, and the results of a study of 34 strains, 23 from human pathologic sources and 11 from other sources, are reported

Enterococci have been found in a great variety of human ailments They appear to be important causal agents in some cases of endocarditis, intestinal disorders, abdominal infections due to injury of the intestinal tract, infections of wounds inflicted during war, and infections of the urinary tract

The following characteristics distinguish the enterococci from other streptococci reaction in serum of group D according to Lancefield's precipitin test, growth at 10 and 45 C, growth in media containing 6 5 per cent sodium chloride, growth in media having an initial pH value of 9 6, and growth in media containing 40 per cent bile

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ANTIBIOTIC ACTIVITY OF THE FATTY-ACID-LIKE CONSTITUENTS OF WHEAT BRAN

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An appreciable part of the antibiotic activity observed in connection with the culturing of an unidentified microorganism on a medium consisting principally of wheat bran and asparagus juice was found to reside in the medium itself   Investigation disclosed that the wheat bran was the source of this activity   Wheat bran has frequently been used as an ingredient in media assayed for antibiotic activity   Because of the possibility of confusing the antibiotic activity naturally present in wheat bran with the activity produced by microorganisms grown on media containing wheat bran, efforts were made to characterize the active factor involved   A hypothesis is presented that antibiotic activity may be formed by the hydrolysis and saponification (by the action of the microorganisms) of fatty constituents contained in the original substrates

EXPERIMENTAL PROCEDURE

Preliminary observations indicated that the constituent or constituents of wheat bran possessing antibiotic activity were extractable with 60 or 95 per cent ethanol, petroleum ether, or diethyl ether, but were not appreciably water-soluble   These extracts, particularly those obtained by the use of petroleum ether or diethyl ether, when saponified with KOH, yielded soaps which had even greater activity on an equivalent basis than the original bran   Extraction of the hydrolyzed material with diethyl ether to obtain the neutral and the acid ether-soluble fractions showed that all of the active material was present in the latter

A quantity of the antibiotically active fraction was obtained by extracting 1,000 grams of wheat bran overnight in a percolator with 2 5 liters of petroleum ether, following which procedure the fraction was drained and washed with an additional 1 5 liters of the ether   These extracts were combined and evaporated almost to dryness on a steam bath   The residue was extracted with aliquots of 95 per cent ethanol, totaling about 300 ml   Three hundred ml of 0 832 N alcoholic KOH were added to the ethanol solution, and the mixture was refluxed for 2 hours   The refluxed solution was concentrated to about 200 ml and then diluted with 1,600 ml distilled water   This solution was then extracted with five 200-ml portions of diethyl ether to remove the "neutral" ether soluble fraction   The alcohol-water solution was then acidified with HCl to pH 2, and the diethyl ether extraction was repeated   After being washed with water, the combined acid-ether extracts were evaporated, from which about 30 g of a brown, oily residue were obtained   This residue, which contained the active material,

was then extracted with 95 per cent ethanol   The ethanol-soluble fraction was removed by centrifuging the suspension of the residue in the ethanol, and a potas sium salt was prepared from the supernatant by the addition of 90 ml of 0 096 N KOH   This solution, after dilution with about 600 ml of water, was shell frozen in round-bottomed boiling flasks and then dried under vacuum from the frozen state   The final yield was 27 g

Tests for antibiotic activity were made on each extract at each step of the separation, and a control containing only the solvent was made on each solvent It was calculated that the final product contained about 95 per cent of the original activity   It was found that the refluxed alkaline alcohol solution was more active than the original petroleum ether extract, which was probably due to the hydroly sis of some of the fats to free fatty acids   The solvent controls showed no activity at the inhibition levels of the active extracts

A modified medium II of Schmidt and Moyer (1944) was used for the bioassay The ingredients of the medium were peptone, 5 g, yeast extract, 1 5 g, beef ex tract, 1 5 g, "N-Z-amine" type B, 2 0 g, glucose, 3 0 g, NaCl, 3 5 g, 500 ml of KH_2PO_4 (15 g per liter) adjusted to pH 7 0 with NaOH, and distilled water to make 1 liter   The medium was placed in bottles of convenient size, usually in the amount required for any one series of assays   The inoculum was grown by transferring it from an agar slant to 100 ml of the medium in a 250-ml Erlenmeyer flask, and incubating it 18 hours at 37 C   The inoculum was added to the me- dium at the rate of 20 ml per liter and the serial dilution set up   Two logarithmic series of 10, 100, 1,000, etc , and 5, 50, 500, etc , were set up for the survey assay during the fractionation   When more accuracy was desired, as in the comparison of solutions of various salts of the fatty acids, series of 120, 200, 300, 400, 600, 800, and 1,200, or 10-fold multiples thereof, were set up   Test tubes 18 by 150 mm in size were used   Each series was made in duplicate and inoculated, and sterile control blanks were set up with each series   The tubes were incubated for 4 hours in a water bath at 37 C   At the end of the incubation period the tubes were sterilized, and the turbidity of each tube was measured in a Klett Summerson colorimeter   The turbidity readings were plotted against the dilu tions on a semilogarithmic paper   A sigmoidal inhibition curve was obtained The point of 50 per cent inhibition as compared with the readings of the anti biotic-free controls was taken as the most accurate measure of degree of in hibition   From the dilution at which this point in the curve occurred the con centration in micrograms per milliliter was calculated   For the comparison of the activity of the wheat bran fraction with some other salts of fatty acids, Staphylococcus aureus (Food and Drug Administration strain 209), Micrococcus conglomeratus (Merck's N Y strain), Streptococcus faecalis (ATCC 7080), and Escherichia coli (Waksman's strain for testing streptomycin) were used as the assay organisms

Potassium laurate, sodium oleate, potassium salt of mixed acids of castor oil, potassium salt of the mixed acids of cottonseed oil,[2] and a sample of potassium

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[2] Samples of these were supplied by Dr Ernest Kester of this laboratory

lmoleate made from methyl lmoleate[2] were used for the purpose of comparing
activities

RESULTS

Extracts obtained by treating separate 10-g samples of wheat bran with 200-ml
portions of water, and with 70 per cent ethanol, produced a 50 per cent inhibition
of *S aureus* at dilutions of 10 and 260, respectively   A comparison with various
other salts of fatty acids readily available is given in table 1   The results in-
dicated that the potassium salt obtained from the wheat bran was considerably
more active than any of the other salts tested, with the exception of potassium
lmoleate, which had about the same activity as the salts of the wheat bran
extracts

TABLE 1

Antibiotic activity of the salts of fatty acids from various sources

FATTY ACID SALTS	CONCENTRATION OF SALTS GIVING 50 PER CENT INHIBITION OF		
	S aureus	*M conglomeratus*	*S faecalis*
	μg per ml	*μg per ml*	*μg per ml*
Potassium laurate	22	16	27
Sodium oleate	23	18	100
Potassium salts of mixed acids of castor oil*	50	38	45
Potassium salts of mixed acids of cotton seed oil*	50	41	48
Potassium salt of acid ether fraction of wheat bran	4	5 5	10
Potassium linoleate	3 5	4 2	6

* These samples were stored laboratory samples, and it is likely that freshly made
samples would have shown higher activity

Results with *E coli* indicated no inhibition within the range tested   In fact,
with the wheat bran salt, a definite stimulation was noted at 300 micrograms per
milliliter   These findings would seem to indicate that these salts would probably
be more active against gram-positive organisms than against gram-negative ones

DISCUSSION

Germicidal and bacteriostatic activity of some fatty acids is well known
Stimulatory action of these and similar materials at certain concentrations also
has been reported   Whether the action will be stimulatory, inhibitory, or germi-
cidal apparently depends upon the kind and concentrations of the materials
added, on the physical and chemical environment, and on the type of organism
employed   In riboflavin assays, an alcoholic extract of fresh liver hydrolyzed
with alkali was found to be strongly inhibitory for *Lactobacillus casei* (Feeney
and Strong, 1942)   Feeney and Strong also found that ether extract of whole
blood was stimulatory at low, and inhibitory at higher, concentrations   Kodicek

[2] Supplied by Dr Gordon Rose of the Enzyme and Phytochemical Research Division of
this bureau

and Worden (1944), also studying factors affecting the riboflavin assay, found that *Lactobacillus helveticus* was inhibited for 24 hours by oleic acid, and for 72 hours or more by linoleic and linolenic acids, when used in concentrations of 160 micrograms per 10 ml of culture, or at 16 parts per million   Avery (1918) was able to suppress the growth of pneumococci and streptococci, while attempting to isolate "*B influenza*," by adding sodium oleate to a hemoglobin medium The activity of various fatty acid soaps was tested by Lamar (1911*a*, 1911*b*, 1912)   Lysis of pneumococci was obtained at comparatively high dilutions of sodium oleate, potassium linoleate, and potassium linolenate   The latter two salts inhibited growth for 1 hour at dilutions up to 1 4,000 and 1 6,000   It was concluded that the action was directly proportional to the degree of unsaturation of the acid

Bergström, Theorell, and Davide (1946) found that the presence of fatty acids in the medium interfered with the oxygen uptake of *Mycobacterium tuberculosis* Di-heptylacetic acid reduced oxygen uptake at a 1 7,000 dilution, whereas the uptake was completely inhibited by oleic acid at 1 10,000, by linoleic acid at 1 15,000, and by linolenic acid at 1 30,000   After extended investigation, Stanley, Coleman, Greer, Sacks, and Adams (1932) concluded that the most active compounds were aliphatic acids which contain from 15 to 18 carbon atoms. They studied the action of chaulmoogra oil and related compounds on *Mycobacterium leprae*, *Mycobacterium tuberculosis*, and other acid-fast bacteria   The most effective acids were good surface tension depressants   This physical property seemed to be more important than the detailed chemical structure   The sodium salts of these acids were found to be effective in dilutions of 1 50,000, or at 20 parts per million

Barton-Wright (1938) reported that, in the fatty fraction of wheat bran, the total combined acids are 84 per cent unsaturated, with an iodine value of 152 It seems safe to assume the presence of a considerable amount of linoleic acid

The separation from wheat bran of a material with antibiotic properties, which is of a fatty acid nature or is closely associated with the fatty acid, has some rather interesting implications   For instance, when wheat bran, which contains fatty acid constituents, is extracted with 70 per cent ethanol, antibiotic activity is obtained in the extract   Also, the possibility no doubt exists that such fatty constituents may be hydrolyzed by the action of the microorganisms and consequently saponified   When the culture is subsequently assayed, water soluble antibiotic activity may be found, and such activity may be attributed to the antibiotic normally produced by the microorganism

Keeping this hypothesis in mind, it might be well to re-examine the first reported by Srinivasa (1944), Mohan *et al* (1946), Moyer and Coghill (1 and Holtman (1945)   For instance, extracts obtained by the extraction of M and Coghill's (1947) wheat bran medium with 70 per cent alcohol at pH 7 5 a 50 per cent inhibition of *S aureus* at 1 200, of *S faecalis* at 1 108, and *conglomeratus* at 1 60   Hence, if an organism capable of hydrolysis of th constituents of the medium were grown, a water-soluble salt having ant activity might be formed, which, when the medium was assayed, could false picture of antibiotic activity produced by the organism   Actual su

, ... , ... order to c ...ain the correct data   Intensive search of the literature might bring to light numbers of instances in which the addition to media of fat- and fatty-acid-containing materials resulted in an increase in antibiotic activity

SUMMARY AND CONCLUSIONS

A fraction having antibiotic properties was extracted from wheat bran   This material has the characteristics of a fatty acid and forms a water-soluble potassium salt which has a comparatively high activity against *Staphylococcus aureus*, *Micrococcus conglomeratus*, and *Streptococcus faecalis*   It was inactive against *Escherichia coli*   When materials of plant or animal origin containing fats or fatty acid constituents are used in making microbiological media, the possibility of these constituents having antibiotic activity, which might be confused with activity produced by microorganisms, should be given consideration

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RIBOFLAVIN PRODUCTION BY MOLDS[1]

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Very little information is at hand regarding the ability of molds to synthesize riboflavin   Few citations pertaining directly to riboflavin production by true molds are available [2]   Pontovich (1943) found as much as 2 mg riboflavin per g of *Aspergillus flavus* mycelium   Tanner et al (1945) determined the quantity of riboflavin in the submerged fermentation media of *Penicillium chrysogenum* The highest value found was 1 36 mg per ml   The primary purpose of this study was to screen several hundred isolates, recently obtained from soil, crop residues, and composts, for their ability to produce riboflavin on a wheat bran substrate

METHODS

Preparation and inoculation of wheat bran   Ten grams of wheat bran and 10 ml of water were thoroughly mixed in 12-oz French squares   The bottles were then plugged with cotton and autoclaved for 60 minutes at 121 C

The mold isolates were carried on potato glucose slants, and the inoculum was prepared as follows Several grams of sterile, moistened wheat bran (in test tubes) were inoculated directly from the agar slants and allowed to sporulate well   Approximately 0 5 g of this dried mold bran served as the inoculum for each bottle   The bottles were incubated horizontally at 30 C until good mycelial growth was obtained (72 to 96 hours)

Riboflavin assay   The dry mold bran was assayed for riboflavin by the *Lactobacillus casei* acid production method of Snell and Strong (1939) as modified by Strong and Carpenter (1942)

RESULTS

The results of the screening tests are presented in table 1   Of the 240 isolates, all were capable of riboflavin synthesis   As will be noted, however, some genera are better able to produce riboflavin than others   The isolates of the genus *Fusarium* are rather outstanding in this respect, as well as are certain of the aspergilli   The most outstanding isolate was a "gold" *Aspergillus* which yielded a value of 5 8 mg riboflavin per 100 g of mold bran

TABLE 1

Riboflavin production by molds

GENUS	NUMBER OF ISOLATES	RIBOFLAVIN PER 100 G MOLD BRAN*			
		0 40–0 49 mg	0.50–0 99 mg	1 00–1 99 m₄	2.00+ g
Aspergillus					
black	47	0	2	39	6
green	19	0	2	17	0
tan	27	0	10	8	9
gold	16	0	2	9	5
misc	4	0	1	2	1
Penicillium					
blue-green	27	0	11	14	2
gray-green	19	0	10	7	2
yellow-green	11	2	5	4	0
compact raised	15	2	5	7	1
Alternaria	4	0	2	2	0
Fusarium	26	0	3	7	16
Hormodendrum	9	1	5	3	0
Rhizopus-Mucors	11	0	6	4	1
Trichoderma	5	0	0	3	2
Total	240	5	64	126	45

* From 0 25 to 0 35 mg riboflavin per 100 g wheat bran before molding

SUMMARY

Of the 240 fungal isolates grown on a wheat bran substrate, all were capable of producing some riboflavin   Forty-five isolates gave values in excess of 2 mg per 100 g of mold bran   Certain isolates of the genera *Fusarium* and *Aspergillus* were particularly outstanding   The highest yield of riboflavin, 5 8 mg per 100 g of mold bran, was obtained from a "gold" *Aspergillus*   It can be concluded that riboflavin synthesis is rather common, at least in the molds studied, and that certain isolates produce riboflavin in amounts sufficient to warrant further study as a biological source of riboflavin

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STUDIES ON THE MODE OF ACTION OF STREPTOMYCIN

II THE NATURE OF A STREPTOMYCIN INHIBITOR OCCURRING IN BRAIN TISSUE AND PLANT EXTRACTS[1]

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The action of streptomycin on *Eberthella typhosa* and *Staphylococcus aureus* was found by Wallace, Rhymer, Gibson, and Shattuck (1945) to be much greater in a poor medium (nutrient broth) than in a good medium (brain heart infusion) An interpretation was made that "there is something present in the brain heart infusion which interferes in some way with the action of streptomycin which is not present in nutrient broth " Similar observations have been reported by others and in some cases different interpretations have been made Berkman, Henry, and Housewright (1947), for example, believe that the resistant individuals in any one test grow out faster in the better medium than they do in the poorer medium and that an interfering substance is not necessarily present Further studies have been made to determine, if possible, the nature of the interfering substances

The streptomycin was prepared at the University of Illinois in the laboratory of H W Anderson and purified and standardized in the laboratory of H E Carter (Carter *et al*, 1945, Loo *et al*, 1945) The unit used is equivalent to the FDA unit, which is equal to one microgram of the free base or 0 84 micrograms of the hydrochloride The crystalline streptomycin was dissolved in the liquid medium under study, thereby eliminating the error that might arise from dilution of the test medium

Eberthella typhosa (Hopkins strain) and *Staphylococcus aureus* (FDA 209) were again used as the test organisms A 24-hour broth culture of the organism was diluted with twice its volume of sterile water for the inoculum, 0 1 ml of this suspension being used in all cases In the tests, plate counts were made at the start and 3, 6, 9, and 24 hours later to determine the numbers of viable organisms present

Because considerable amounts of brain heart infusion were to be used in the studies, the use of Difco brain heart infusion seemed desirable for most consistent results The brain heart infusion used up to this time was prepared in the laboratory by the authors and did not have any inorganic salts added to it Since the Difco medium contained both sodium chloride and disodium phosphate, a series of tests were run to see whether the salts in the Difco medium would have any effect upon the streptomycin The two media gave almost identical results in these tests, so it was concluded that sodium chloride and disodium phosphate in

the amounts present in Difco brain heart infusion would not interfere with the action of streptomycin

Since the first published report considered only amounts of streptomycin which would decrease the numbers of microorganisms, this work was continued to determine the concentration of streptomycin necessary to destroy all organisms It was found that approximately 4 1 units of streptomycin per ml of culture medium would destroy all the added cells of *Eberthella typhosa* in nutrient broth, and that approximately 1 unit of streptomycin per ml of culture medium would destroy all the added cells of *Staphylococcus aureus* in nutrient broth   In brain heart infusion the results were entirely different   A concentration of streptomycin of 50 units per ml of medium failed to destroy all the cells of *Eberthella typho* , and prevent growth of the organism in the medium   Twenty-five units per ml did not destroy the cells of *Staphylococcus aureus* in the brain heart infu ion In this particular study the plate counts were used to follow the killing action of streptomycin   The results again indicate that a protective substance for th organism under test or an antagonistic substance to streptomycin is present in the brain heart infusion

In order to determine the nature of this substance Wolf's (1945) "casamino acid" medium was used as a base medium for further study   This medium is constant in composition and is reproducible   The complete dehydrated medium was obtained from Difco Laboratories and also vitamin-free casamino acids for the preparation of the complete medium [2]   In this medium it was found that '0 units of streptomycin per ml of culture and 2 6 units of streptomycin per ml of culture would destroy all the added cells of *Eberthella typhosa* and *Staphylococcus aureus*, respectively   The amount of streptomycin necessary here is considerably more than that needed for destruction in the nutrient broth, but very much b than that needed in the brain heart infusion   This may be interpreted as indicating that the interfering substance or substances are present in the casamino acid medium but not in the same concentration as they are in brain heart infusion   It seemed desirable first to determine the effect of the constituent in the complete casamino acids medium   Uracil, thiamine hydrochloride, and nicinamide are present in the medium, so the complete casamino acid medium was compared with casamino acid medium minus thiamine hydrochloride, casamino acid medium minus niacinamide, casamino acid medium minus uracil, and casamino acid medium minus thiamine hydrochloride, niacinamide, and uracil The results were all similar, indicating that the constituents present except the casamino acids were not affecting, in any way, the action of the streptomycin Some inorganic salts are present in the casamino acids medium, but since an earlier study had indicated the unimportance of sodium chloride and dihydrogen phosphate it was thought advisable to postpone study of the salt content and to examine the casamino acids or the nitrogen-containing constituents of culture media for the interfering factor

At this point it was decided to change the test procedure   Because a g ''

many substances were to be tested and the plate counting procedure was tedious and time-consuming, it was thought to be more advantageous to change from a measurement of numbers of bacteria present by plate counting to one of presence or absence of turbidity   The culture tubes were examined for presence or absence of turbidity as compared with an untreated culture in the test medium Some checks were made comparing this type of reading results with plate counts and it was found that sufficiently accurate readings could be made

Effect of adding commercial peptones to base medium   The available commercial bacteriological peptones were then added to the casamino acid medium

TABLE 1

Action of streptomycin on Eberthella typhosa in casamino acid medium with addition of different peptones

PEPTONES	CONCENTRATION OF STREPTOMYCIN IN UNITS PER ML																	
	0	3 3	6 7	10 0	13 3	16 7	20 0	21 7	23 3	25 0	26 7	28 3	30 0	31 7	33.3	35	36 7	38.3
0 5% Liver fraction L (W)	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
1 0% Phytone (B B L)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
1 0% Neopeptone (D)	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
1 0% Peptone (D)	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
1 0% Proteose peptone III (D)	+	+	+	+	+	+	−	+	+	−	+	−	−	−	−	−	−	−
1 0% Trypticase (B B L)	+	+	+	+	+	−	+	+	+	+	+	−	+	−	−	−	−	−
1 0% Proteose peptone (D)	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
1 0% Tryptose (D)	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−
1 0% Tryptone (D)	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−
1 0% Casamino acids (D)	+	+	+	+	+	−	+	+	+	+	+	+	−	+	−	−	−	−
Casamino acid medium (D)	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

(W) = Wilson Laboratories   (B B L) = Baltimore Biological Laboratory   (D) = Difco Laboratories

+ = turbidity   − = no turbidity

and the effect on streptomycin noted   With *Eberthella typhosa* phytone (B B L), liver fraction L (Wilson), trypticase (B B L), and tryptone (Difco) had an inhibitory effect on the action of streptomycin, with the degree of activity in the order listed   Tryptone (Difco), proteose peptone no 3 (Difco), peptone (Difco), proteose peptone (Difco), and neopeptone (Difco) all inhibited streptomycin slightly   Phytone, liver fraction L, and neopeptone gave the greatest inhibitory action with *Staphylococcus aureus,* whereas the remainder of the peptones had only slight effect   Tables 1 and 2 give these results in detail

The results at this stage of the investigation all pointed toward the presence of some factor in the nitrogen-containing constituents of culture media which interfered with the action of streptomycin   It was present in varying amounts

but v as greatest in brain heart infusion medium   Of the bacteriological p -. tones, it \ as present in greatest amount in phytone, a peptone made from p!...' proteins   Because the brain heart infusion had so much activity, an intensi,- study was then made of it in an attempt to learn more about the interfe in, substance

Adsorption of brain heart infusion with activated carbon   Brain heart inu... a was treated with "darco" once and tested for action   It was found to contain all of its activity   It was then treated with darco five times and tested, again showing no decrease in its ability to inhibit the action of streptomycin   It

TABLE 2

Action of streptomycin on Staphylococcus aureus in casamino acid medium with the addition of different peptones

PEPTONES	CONCENTRATION OF STREPTOMYCIN IN UNITS PER ML																	
	0	1 7	3 3	5 0	6 7	8 0	9 0	10 0	11 0	12 0	13 0	14 0	15 0	16 0	17 0	18 0	19 0	19 0
0 5% Liver fraction L (W)	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−
1 0% Phytone (B B L)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	−
1 0% Neopeptone (D)	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
1 0% Peptone (D)	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1 0% Proteose peptone III (D)	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
1 0% Trypticase (B B L)	+	+	+															
1 0% Proteose peptone (D)	+	+	−					−	−	−	−	−	−	−	−	−	−	−
1 0% Tryptose (D)	+	+	±					−	−	−	−	−	−	−	−	−	−	−
1 0% Tryptone (D)	+	+	+					−	−	−	−	−	−	−	−	−	−	−
1 0% Casamino acid (D)	+	+	+					−	−	−	−	−	−	−	−	−	−	−
Casamino acid medium (D)	±	+	−					−	−	−	−	−	−	−	−	−	−	−

(W) = Wilson Laboratories   (B B L) = Baltimore Biological Laboratory   (D) = Difco Laboratories
± = turbidity   − = no turbidity

seems rather definite, therefore, that no vitamin or any substance \   adsorb to the carbon could account for this action

Hydrolysis of brain heart infusion   After hydrolysis with uy heart infusion would not support growth of *Eberthella typhosa*, so not be measured directly   *Staphylococci*   grow on *' brain heart infusion, but not well   Casa   ..cil, tl am ride, and niacinamide were added to the   in the am' present in casamino acid medium, and   *Eberthell* *Staphylococcus aureus* was obtained   ,,   hydi. niacinamide were added, the same results alone   Liver fraction L was added to the ' gave good growth   Phytone was hydroly

those obtained with the hydrolyzed brain heart infusion   The activity of the
hydrolyzates in interfering with streptomycin action closely paralleled the growth
activity   Apparently the growth factor and the streptomycin-interfering factor
are both destroyed by this process, or perhaps the two activities are caused by
one substance which is destroyed

Study of brain and heart infusions separately   Difco Laboratories prepare a
heart infusion broth culture medium containing infusion from beef heart, tryp-
tose, and sodium chloride   This medium was tested for its streptomycin-in-
hibiting activity and was found to have none, which indicates very strongly that
the activity is present in the brain infusion   Consequently a brain infusion was
obtained from two sources, the Difco Laboratories and H E Carter, University
of Illinois, and both were studied for their activity   The two infusions gave
parallel results, indicating, as was suspected, that the activity was present in
rather large amounts in brain tissue

TABLE 3

EXTRACTION	BRAIN HEART INF		PHYTONE	
	E typhosa	S aureus	E typhosa	S aureus
Methanol extract	++	++	+	+
Residue of methanol extract	+	+	++	++
Ether precipitate of methanol extract	++	++		
Ether-soluble portion of methanol extract	+	+		

+ = presence of antistreptomycin activity

Extraction of brain heart infusion and phytone   Brain heart infusion and phy-
tone were then extracted with methanol and the extract and residue were both
tested for activity   When an equal volume of ether was added to the methanol
extract of brain heart infusion a precipitate was formed, so the precipitate and
the ether-soluble part were tested   Table 3 gives the results of these studies
The results were quite sharp and indicate that in the brain heart infusion, al-
though the extraction was not complete, most of the activity was present in the
methanol extract   The ether precipitate also carried most of the activity from
the methanol extract   In the phytone the activity was greatest in the residue
of the methanol extract, which indicates that the substances in brain heart in-
fusion and phytone are not identical or that something interferes with their
extraction by methanol

Hydrolysis of methanol extract   The methanol extract of brain heart infusion
was hydrolyzed for 1 and for 4 days and tested for activity   It was found that
some activity was destroyed in 1 day and that all activity was destroyed in 4
days

Dialysis of brain heart infusion   Brain heart infusion was dialyzed in a col-
lodion membrane, and the active substance completely dialyzed through the
membrane in running water within 3 hours

The latter tests required an assay procedure which was developed as follo--
The substance to be assayed was dissolved in water so that 1 ml contained 125
mg of the substance   This solution was added to 4 test tubes in 25 mg, 12 5 mg,
6 25-mg, and 3 125-mg amounts   Each of the tubes was then made up to th
2-ml volume with distilled water   To each were then added 2 5 ml of doub-
strength casamino acid medium, and the whole was sterilized   One hundred
and twenty-five units of streptomycin contained in 0 5 ml of water and one drop
of a 24-hour broth culture of *Eberthella typhosa* were added to each of the 4 tub-
When *Staphylococcus aureus* was used, each 0 5 ml of the antibiotic solution con-
tained 31 25 units   All tubes were incubated for 15 hours at 37 C and the pres-
ence or absence of turbidity was observed   Brain heart infusion cultures were
assayed at the same time as a control for comparison

DISCUSSION AND CONCLUSIONS

Antibacterial activity of streptomycin has been shown to be greatly influenced
by the composition of the medium in which it is acting   A brain heart infusion
shows great ability to inhibit the action of streptomycin   Certain peptone,
especially phytone, a peptone made from plant proteins, also have this ability
but to a lesser degree   Studies have indicated that the activity is due to some-
thing which can be extracted from the media, thus indicating that a speci-
substance or group of substances is responsible for the inhibitory action   Tests
have shown that brain tissue contains large amounts of an active sub tance
Greater activity was observed with the extract of brain than with the phyton-
Further studies are being made to determine its nature

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THE ACTION OF PHENYLMERCURIC NITRATE

IV THE ABILITY OF SULFHYDRYL COMPOUNDS TO PROTECT AGAINST THE GERMICIDAL ACTION OF BASIC PHENYLMERCURIC NITRATE

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In earlier papers it was shown that yeast extracts have the ability to antagonize the inhibitory action of basic phenylmercuric nitrate on the growth of yeast and bacteria (Cook and Kreke, 1943, Thomas, Fardon, Baker, and Cook, 1945), on the respiration of yeast (Cook and Kreke, 1943), on the growth of molds (Cook and Kreke, 1940), and on the respiration (Cook, Kreke, Eilert, and Sawyer, 1942) and growth (Thomas, Fardon, Baker, and Cook, 1945) of skin

In attempts to elucidate these findings it was demonstrated that this germicide depresses the activity of cytochrome oxidase, succinic, lactic, and glucose dehydrogenases, and catalase (Cook, Kreke, McDevitt, and Bartlett, 1946) Further experimentation showed that the depression of yeast respiration and of cytochrome oxidase activity by phenylmercuric nitrate, as measured by the Warburg respirometer, could be prevented but not reversed by the sulfhydryl compounds cysteine, homocysteine, and glutathione, whereas cystine and methionine, as well as a number of amino acids containing other reactive groups, were ineffective as protecting agents (Cook, Perisutti, and Walsh, 1946, Cook and Perisutti, 1947)

The present paper demonstrates that the inhibition of the growth of *Escherichia coli*, *Eberthella typhosa*, and *Staphylococcus aureus* by basic phenylmercuric nitrate can be antagonized by the addition of sulfhydryl-containing compounds Efforts to reverse the inhibition after exposure of *Escherichia coli* to the germicide have been unsuccessful

EXPERIMENTAL PROCEDURES

Solid medium Filter disc method In preliminary experiments nutrient agar containing cysteine in concentrations ranging from 1.5×10^{-6} M to 6.0×10^{-4} M was prepared After sterilization and subsequent cooling to 42 C, the medium was poured over 0 1 ml of a 48-hour nutrient broth culture of the test organism in sterile petri dishes After solidification of the agar, filter paper discs dipped in phenylmercuric nitrate solutions of a concentration range from 1.5×10^{-6} M to 1.5×10^{-4} M were placed on the surface of the agar The control medium was nutrient agar without cysteine Zones of inhibition were measured after 18 hours' incubation at 37 C There was evidence of antagonism as shown in table 1 This method does not allow comparison of effective concentrations of germicide and cysteine since the actual concentration of phenylmercuric nitrate in contact with cysteine in the medium depends upon diffusion and hence is unknown

In all subsequent experiments the sulfur-containing compounds (cysteine, homocysteine, glutathione, cystine, or methionine) were mixed with the phenyl mercuric nitrate, and the mixture was allowed to stand 5 minutes to react. reaction   The filter discs were dipped in the solution and placed on the surface of the agar   In both of these methods the diameter of the filter paper disc was 12 7 mm so that a 13-mm zone of inhibition represents only trace activity and less than this value is represented in the tables as 0 inhibition

TABLE 1

Growth of organisms with cysteine incorporated in medium and basic phenylmercuric nitrate (PMN) applied to filter paper discs

PMN CONC (M)	CYSTEINE CONC (M)	INHIBITION ZONE, MM IS IN		
		E coli	E typhosa	S aureus
1 5 × 10⁻⁴	0	21	23	31
1 5 × 10⁻⁵	0	17	0	23
1 5 × 10⁻⁶	0	0	0	19
1 5 × 10⁻⁴	1 5 × 10⁻⁶	19	19	31
1 5 × 10⁻⁵	1 5 × 10⁻⁶	0	0	23
1 5 × 10⁻⁶	1 5 × 10⁻⁶	0	0	17
1 5 × 10⁻⁴	1 5 × 10⁻⁵	19	17	31
1 5 × 10⁻⁵	1 5 × 10⁻⁵	0	0	23
1 5 × 10⁻⁶	1 5 × 10⁻⁵	0	0	16
1 5 × 10⁻⁴	1 5 × 10⁻⁴	17	17	31
1 5 × 10⁻⁵	1 5 × 10⁻⁴	0	0	23
1 5 × 10⁻⁶	1 5 × 10⁻⁴	0	0	16
1 5 × 10⁻⁴	3 0 × 10⁻⁴	16	19	31
1 5 × 10⁻⁵	3 0 × 10⁻⁴	0	0	23
1 5 × 10⁻⁶	3 0 × 10⁻⁴	0	0	16
1 5 × 10⁻⁴	6 0 × 10⁻⁴	16	17	31
1 5 × 10⁻⁵	6 0 × 10⁻⁴	0	0	23
1 5 × 10⁻⁶	6 0 × 10⁻⁴	0	0	16

* Filter disc diameter, 12 7 mm   A 13-mm zone of inhibition therefore represents only trace activity, and less than this is represented in tables as 0 inhibition

Liquid medium   Nutrient broth was prepared containing both phenyl mercuric nitrate and cysteine in the same concentrations as were used in the experiments with the solid media   The inoculum was prepared by diluting 0 1 ml of a 48-hour broth culture of the test organism in 500 ml of sterile physiological saline and using 0 1 ml of this diluted culture to seed the tubes   Growth was determined after 18 hours' incubation at 37 5 C by centrifugation in Hopkins' tubes at 2,000 rpm for 20 minutes

All experiments, employing both solid and liquid media, were accompanied by controls containing the sulfur compounds in the appropriate concentration

Growth ∪ organisms ∪∪ ∪∪∪ ∪ ∪, ∪
nitrate (PMN) mixed and applied to filter paper disc

PMN CONC (M)	ANTAGONIST CONC (M)	INHIBITION ZONE, MM,* 18 HR		
		E coli	E typhosa	S aureus
Cysteine†				
1 5 × 10⁻⁴	0	23	23	31
1 5 × 10⁻⁵	0	17	0	21
1 5 × 10⁻⁶	0	0	0	17
1 5 × 10⁻⁴	1 5 × 10⁻⁶	23	23	31
1 5 × 10⁻⁵	1 5 × 10⁻⁶	15	0	21
1 5 × 10⁻⁶	1 5 × 10⁻⁶	0	0	15
1 5 × 10⁻⁴	1 5 × 10⁻⁵	20	21	31
1 5 × 10⁻⁵	1 5 × 10⁻⁵	14	0	19
1 5 × 10⁻⁶	1 5 × 10⁻⁵	0	0	0
1 5 × 10⁻⁴	1 5 × 10⁻⁴	20	19	31
1 5 × 10⁻⁵	1 5 × 10⁻⁴	0	0	18
1 5 × 10⁻⁶	1 5 × 10⁻⁴	0	0	0
1 5 × 10⁻⁴	3 0 × 10⁻⁴	18	17	29
1 5 × 10⁻⁵	3 0 × 10⁻⁴	0	0	18
1 5 × 10⁻⁶	3 0 × 10⁻⁴	0	0	0
1 5 × 10⁻⁴	6 0 × 10⁻⁴	17	16	28
1 5 × 10⁻⁵	6 0 × 10⁻⁴	0	0	17
1 5 × 10⁻⁶	6 0 × 10⁻⁴	0	0	0
Homocysteine				
1 5 × 10⁻⁴	0	25	25	31
1 5 × 10⁻⁵	0	17	0	23
1 5 × 10⁻⁶	0	0	0	17
1 5 × 10⁻⁴	1 5 × 10⁻⁶	25	25	31
1 5 × 10⁻⁵	1 5 × 10⁻⁶	17	0	23
1 5 × 10⁻⁶	1 5 × 10⁻⁶	0	0	15
1 5 × 10⁻⁴	1 5 × 10⁻⁵	25	23	31
1 5 × 10⁻⁵	1 5 × 10⁻⁵	15	0	21
1 5 × 10⁻⁶	1 5 × 10⁻⁵	0	0	0
1 5 × 10⁻⁴	1 5 × 10⁻⁴	23	19	29
1 5 × 10⁻⁵	1 5 × 10⁻⁴	15	0	21
1 5 × 10⁻⁶	1 5 × 10⁻⁴	0	0	0
1 5 × 10⁻⁴	3 0 × 10⁻⁴	23	19	27
1 5 × 10⁻⁵	3 0 × 10⁻⁴	0	0	19
1 5 × 10⁻⁶	3 0 × 10⁻⁴	0	0	0
1 5 × 10⁻⁴	6 0 × 10⁻⁴	19	17	27
1 5 × 10⁻⁵	6 0 × 10⁻⁴	0	0	19
1 5 × 10⁻⁶	6 0 × 10⁻⁴	0	0	0

TABLE 2—*Continued*

PMN CONC (M)	ANTAGONIST CONC (M)	INHIBITION ZONE MM IS ER		
		E coli	E typhosa	S au ...
Glutathione				
$1\ 5 \times 10^{-4}$	0	24	21	29 5
$1\ 5 \times 10^{-5}$	0	14	0	22 0
$1\ 5 \times 10^{-6}$	0	0	0	15 0
$1\ 5 \times 10^{-4}$	$1\ 5 \times 10^{-6}$	24	21	29
$1\ 5 \times 10^{-5}$	$1\ 5 \times 10^{-6}$	14	0	21
$1\ 5 \times 10^{-6}$	$1\ 5 \times 10^{-6}$	0	0	15
$1\ 5 \times 10^{-4}$	$1\ 5 \times 10^{-5}$	24	20	29
$1\ 5 \times 10^{-5}$	$1\ 5 \times 10^{-5}$	13	0	16
$1\ 5 \times 10^{-6}$	$1\ 5 \times 10^{-5}$	0	0	0
$1\ 5 \times 10^{-4}$	$1\ 5 \times 10^{-4}$	24	18	29
$1\ 5 \times 10^{-5}$	$1\ 5 \times 10^{-4}$	13	0	16
$1\ 5 \times 10^{-6}$	$1\ 5 \times 10^{-4}$	0	0	0
$1\ 5 \times 10^{-4}$	$3\ 0 \times 10^{-4}$	22	16	20
$1\ 5 \times 10^{-5}$	$3\ 0 \times 10^{-4}$	0	0	15
$1\ 5 \times 10^{-6}$	$3\ 0 \times 10^{-4}$	0	0	0
$1\ 5 \times 10^{-4}$	$6\ 0 \times 10^{-4}$	20	15	20 5
$1\ 5 \times 10^{-5}$	$6\ 0 \times 10^{-4}$	0	0	14 0
$1\ 5 \times 10^{-6}$	$6\ 0 \times 10^{-4}$	0	0	0

* Filter disc diameter, 12 7 mm   A 13-mm zone of inhibition therefore represents only trace activity and less than this is represented in tables as 0 inhibition

† $7\ 5 \times 10^{-5}$ M PMN was completely antagonized for *E coli* by $6\ 0 \times 10^{-4}$ M cysteine and for *E typhosa* by $1\ 2 \times 10^{-3}$ M cysteine (control zones, 19 mm) $1\ 5 \times 10^{-5}$ M PMN was completely antagonized for *S aureus* by $1\ 2 \times 10^{-3}$ M cysteine

and omitting the mercurial   In the concentrations used, the compounds thus added did not affect the growth of the bacteria

The test organisms were *Esherichia coli* ATCC no 730, *Eberthella typhosa* ATCC no 7251, and *Staphylococcus aureus*, ATCC no 152

The basic phenylmercuric nitrate ($C_6H_5HgNO_3$ C_6H_5HgOH) was obtained from The Hamilton Laboratories, Inc , glutathione from the Schwarz Laboratories Inc , and $l(+)$cysteine hydrochloride, $l(-)$cystine, dl-homocysteine, and dl-methionine from General Biochemicals, Inc

RESULTS

The data in table 1 demonstrate an antagonism between cysteine in the medium and phenylmercuric nitrate on the filter paper disc as shown by the diminution in the zones of inhibition surrounding the discs   As mentioned earlier, this method does not permit comparison of effective concentrations of

in favor of dipping the discs in mixtures of the germicide and —SH compound   In table 2 are shown the effects by this method of the sulfhydryl-containing compounds cysteine, homocysteine, and glutathione on the growth-inhibiting activity of phenylmercuric nitrate There is a marked difference in the sensitivity of the individual test organism to the germicide as shown by the zones of inhibition in the control group   The greater sensitivity of *Staphylococcus aureus* to the mercurial is in conformity with the findings of Weed and Ecker (1931) and Birkhaug (1933)   Within

TABLE 3

Growth of organisms with cysteine and basic phenylmercuric nitrate (PMN) in nutrient broth

PMN CONC. (M)	CYSTEINE CONC (M)	GROWTH ML HOPKINS TUBES, 18 HR		
		E coli	E typhosa	S aureus
0	0	0 023	0 002	0 002
$1\ 5 \times 10^{-4}$	0	0	0	0
$1\ 5 \times 10^{-5}$	0	0 013	0 001	0
$1\ 5 \times 10^{-6}$	0	0 023	0 002	0
$1\ 5 \times 10^{-4}$	$1\ 5 \times 10^{-6}$	0	0	0
$1\ 5 \times 10^{-5}$	$1\ 5 \times 10^{-6}$	0 010	0 001	0
$1\ 5 \times 10^{-6}$	$1\ 5 \times 10^{-6}$	0 023	0 002	0
$1\ 5 \times 10^{-4}$	$1\ 5 \times 10^{-5}$	0	0	0
$1\ 5 \times 10^{-5}$	$1\ 5 \times 10^{-5}$	0 012	0 001	0
$1\ 5 \times 10^{-6}$	$1\ 5 \times 10^{-5}$	0 023	0 002	0
$1\ 5 \times 10^{-4}$	$1\ 5 \times 10^{-4}$	0	0	0
$1\ 5 \times 10^{-5}$	$1\ 5 \times 10^{-4}$	0 010	0 001	0
$1\ 5 \times 10^{-6}$	$1\ 5 \times 10^{-4}$	0 023	0 002	0 002
$1\ 5 \times 10^{-4}$	$3\ 0 \times 10^{-4}$	0	0	0
$1\ 5 \times 10^{-5}$	$3\ 0 \times 10^{-4}$	0 014	0 002	0
$1\ 5 \times 10^{-6}$	$3\ 0 \times 10^{-4}$	0 023	0 002	0 002
$1\ 5 \times 10^{-4}$	$6\ 0 \times 10^{-4}$	0	0	0
$1\ 5 \times 10^{-5}$	$6\ 0 \times 10^{-4}$	0 018	0 002	0 001
$1\ 5 \times 10^{-6}$	$6\ 0 \times 10^{-4}$	0 023	0 002	0 002

experimental error, the sulfhydryl-containing compounds are equally effective in overcoming the action of the germicide   In contrast, the compounds in which the —SH group is covered (cystine and methionine) were found to be ineffective, and therefore the results of experiments with these compounds have not been tabulated

In table 3 is demonstrated the action of cysteine and phenylmercuric nitrate on the growth of the organisms in nutrient broth   Again, antagonism is evident

DISCUSSION

These experiments show that phenylmercuric nitrate inhibition of the growth of *Escherichia coli, Eberthella typhosa,* and *Staphylococcus aureus* can be dimin-

ished or prevented by the combination of the germicide with the sulfhydryl containing compounds cysteine, glutathione, and homocysteine, but not with cystine and methionine

The reaction between the —SH group and the germicide can be demonstrated, since the nitroprusside test for the sulfhydryl group becomes negative in the test tube when the molar ratio of sulfhydryl compound germicide is greate, than 2 1, as required by theory for the reaction with both mercury atoms of basic phenylmercuric nitrate ($C_6H_5HgNO_3$ C_6H_5HgOH)

In the filter disc experiments, the sulfhydryl compounds at a concentration of $6 0 \times 10^{-4}$ M did not completely overcome the effects of the highest concen tration of phenylmercuric nitrate ($1 5 \times 10^{-4}$ M), although the germicidal effectiveness of this concentration of mercurial was reduced for all organisms The inhibition of *Eberthella typhosa* by $7 5 \times 10^{-6}$ M phenylmercuric nitrate was offset completely by $1 2 \times 10^{-3}$ M cysteine Inhibition of *Escherichia coli* by $1 5 \times 10^{-5}$ M phenylmercuric nitrate could be completely offset by $3 0 \times 10^{-4}$ M or greater concentrations of the sulfhydryl compounds *Staphylococcus aureus* was the most sensitive of the organisms to the germicide, and it was possible to obliterate the activity of $1 5 \times 10^{-6}$ M phenylmercuric nitrate by $1 5 \times 10^{-5}$ M or greater concentrations of the sulfhydryl compounds Thus, in the filter disc determinations, 10 or more moles (5 or more equivalents) of sulfhydryl com pound per mole of phenylmercuric nitrate were required for suppression of activity In nutrient broth, from 20 to 100 moles of cysteine per mole of mer curial (10 to 50 equivalents) were necessary to secure antagonism

In the work on yeast respiration and on enzymes (Cook, Perisutti, and Walsh, 1946, Cook and Perisutti, 1947, and unpublished data) it was also observed that concentrations of sulfhydryl compounds several times greater than the theoreti cal were required for the antagonism of phenylmercuric nitrate Fildes (1940) and Cavallito, Bailey, Haskell, McCormick, and Warner (1945) made similar observations for the antagonism of $HgCl_2$ toxicity by sulfhydryl compounds

These findings suggest that the organic mercurial, basic phenylmercuric nitrate, like mercuric chloride and certain of the natural antibiotics, may be presumed to react with essential —SH groups in the microorganism, supposedly in enzyme systems Although such action probably occurs, as in the inhibition of succinic dehydrogenase, the previously observed inhibition by phenylmer curic nitrate of such enzymes as cytochrome oxidase and catalase (Cook, Irck', McDevitt, and Bartlett, 1946), which have been shown not to require —SH groups for their functioning (Barron and Singer, 1945), and the inability of sulfhydryl compounds to reverse the depression of cytochrome oxidase and yeast respiration by phenylmercuric nitrate (Cook and Perisutti, 1947) have s gested that the germicide may not be specific for —SH groups, but may also react with other active groups in the enzyme protein Consonant with the suggestions is the failure of attempts by the present authors to reverse the in hibiting effects of phenylmercuric nitrate on *Escherichia coli* in broth by se sequent addition (after 0 5 to 6 hours) of as much as 50 equivalents of cysteine or glutathione The reversal experiments, however, leave much to be d

y y ' "   '   " '   (above $5 \times 10^{-7} M$)
in themselves were found to be inhibitory to the organisms   Sahyun *et al*
(1936) also reported that high concentrations of cysteine inhibited the growth
of *Eschcrichia coli* in a synthetic medium   Other evidence suggests that phenyl-
mercuric nitrate may be firmly bound to yeast cells   For example, it was
impossible to reverse the respiratory depressing effects of the mercurial on yeast
respiration with yeast extract (Cook and Kreke, 1943), nor could the respiration
of yeast be restored by washing the phenylmercuric nitrate from the cells after
a 15-minute exposure (Cook and Perisutti, unpublished)

SUMMARY

The growth-inhibiting action of basic phenylmercuric nitrate on *Escherichia
coli, Eberthella typhosa,* and *Staphylococcus aureus* can be antagonized by the
sulfhydryl-containing compounds cysteine, homocysteine, and glutathione, but
not by cystine and methionine

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A BACTERIAL VIRUS FOR ACTINOMYCES GRISEUS[1]

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Attack by bacterial viruses on members of the genus *Actinomyces* has been reported in only a few instances, but bacteriophages which attack *Eumycetes* are prevalent Many industrial processes which employ bacteria are subject to bacteriophage infestations The isolation of bacteriophage from soil and sewage may be easily accomplished, but soil cannot be considered an abundant source of actinophage Isolations of actinomycetes are usually made from fertile soils, however, evidence of phage action is seldom noted A transmissible and filterable lytic agent, which attacks actinomycetes, was reported by Wieringa and Wiebols (1936) This particular phenomenon may be explained as being due to the action of a polyvalent actinophage which initiated lysis not only of the parent culture but also of several other species There are other reports of lysis of *Actinomyces* for which actinophages could not be demonstrated However, the methods used would fail to demonstrate the actinophage for *Actinomyces griseus* (Dmitrieff and Soutéeff, 1936, Katznelson, 1940)

Accompanying the recent large-scale industrial utilization of actinomycetes for the production of antibiotic substances, study of the group has been intensified (Schatz, Bugie, and Waksman, 1944, Porter, 1946) The accumulative generations of growth of the cultures, constantly subject to chance contamination through faulty air filtration or insufficiently sterile laboratory and plant equipment and through errors in techniques, have made it highly probable that actinophages would be rediscovered for actinomycetes In fact, a recent report indicated that an actinophage has been isolated from the streptomycin fermentation (Saudek and Colingsworth, 1947)

EXPERIMENTAL WORK

We have observed an actinophage in laboratory cultures of *A griseus* which were exposed to laboratory air for a 24-hour period Moreover, outbreaks have occurred in a streptomycin production plant, located about 500 miles distant from the research laboratory First recognition of the actinophage occurred in laboratory shake flasks *A griseus* cultures, which had developed under submerged conditions for 24 hours from a 10 per cent vegetative inoculum, were changed to stationary incubation conditions and the cotton plugs removed Thin pellicles developed which showed evidence of plaque formation similar to that usually associated with bacteriophage development The cultures were

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[1] Throughout this paper the designation *Actinomyces* has been used to conform with the fifth edition of *Bergey's Manual of Determinative Bacteriology* In each case, the organism referred to may be classified under the terminology proposed by Waksman and Henrici (1943) as *Streptomyces*

filtered through ultrafine fritted glass filters, and the filtrates proved to be free from bacterial or actinomycete contamination   The filtrate, when added to a newly inoculated submerged culture of *A griseus*, prevented initiation of growth
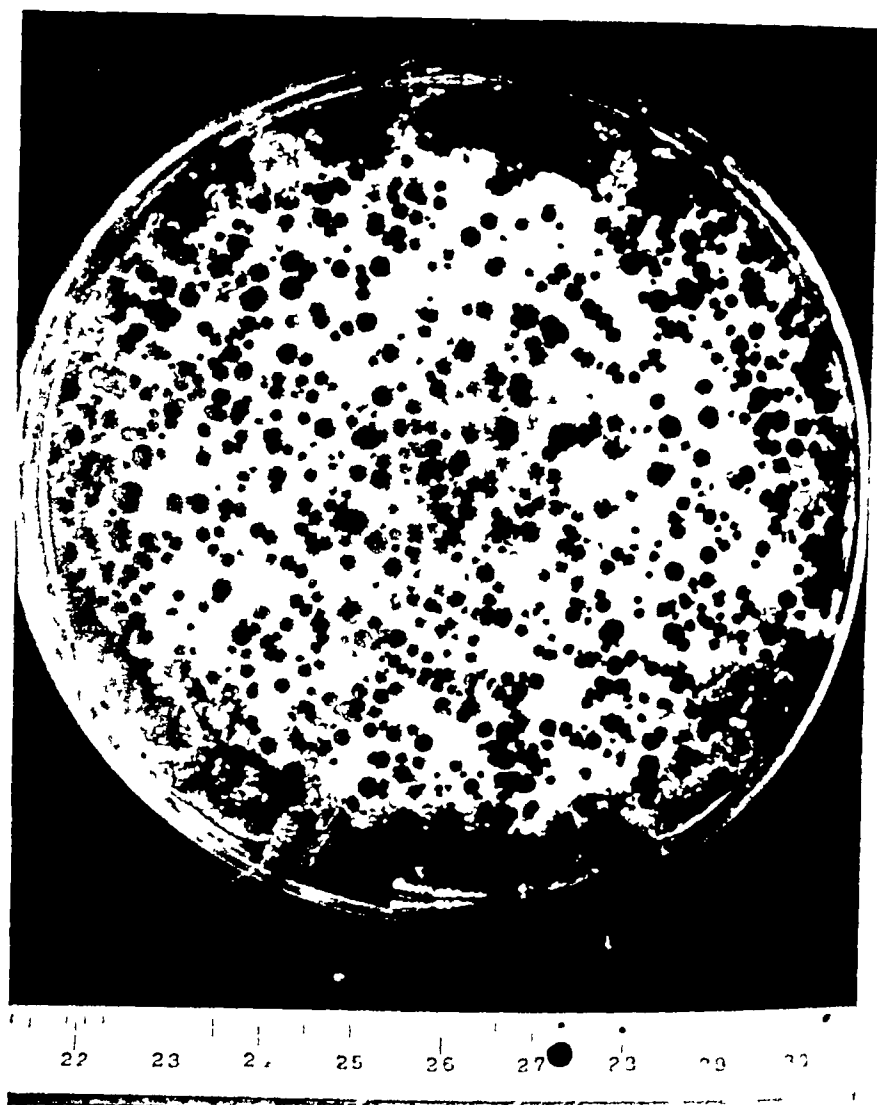
Fig 1 The Formation of Plaques on a Petri Dish Culture

Various dilutions of the filtrate were placed on an agar medium with *A gri* spores   The typical "moth-eaten" cultures, characteristic of bacteriophage contamination, developed within 24 hours   Plaques did not spread during additional incubation   Within 48 hours the *A griseus* growth between the plaques had sporulated and counts of the plaques could be made with a

(figure 1)   The filtrate from the culture in ꞵ ꞏ ꞏ ꞇꞪꞇ ꞮꞮꞯꞯ ꞷ ꞷꞷ tinophage was noted contained 55,000,000 plaque-forming units per ml   A few resistant cultures of *A   griscus* developed when exposed to high concentrations of the actinophage

Actinophage infestations of *A   griscus* in a streptomycin production plant have occurred   In each case simultaneous bacterial contamination or other factors indicated an outside source of the actinophage   No evidence has been found that the actinophage was derived from stock cultures of *A   griseus*

Multiplication of actinophage   The lytic agent was carried through several cultures of *A   griscus* in series and initiated lysis in each instance   To prove transmissibility of the agent, 0 01 ml of a bacteria-free filtrate was transferred to 50 ml of *A   griscus* culture   After 24 hours of submerged growth, the lysed culture was filtered and 0 01 ml of filtrate added to a new culture   The transfers, with filtrations between each, were continued for a total of six cultures

TABLE 1

Multiplication of actinophage

TRANSFER	ACTINOPHAGE PER ML	MULTIPLICATION FACTOR	
		Individual transfers	Accumulative
Phage inoculum	20,000,000,000		
1st transfer	32,800,000,000	8,200	8,200
2nd transfer	100,000,000,000	16,000	131×10^6
3rd transfer	36,000,000,000	1,800	236×10^9
4th transfer	48,000,000,000	6,600	156×10^{13}
5th transfer	64,000,000,000	6,600	103×10^{17}
6th transfer	9,600,000,000	735	75×10^{20}
Control *A griseus*	<10		

Filtrates from each flask were saved and plated by the plaque method for the determination of numbers of actinophage   These determinations (table 1) prove that the agent is transmissible and multiplies after each transfer   For each plaque-forming particle added to the first *A   griseus* culture in the series, a total of 75×10^{20} particles had been produced on completion of the sixth transfer

Actinophage-susceptible strains of A   griseus   Most bacteriophages are specific in activity against a single strain of a species   Actinophage was first isolated from cultures of *A   griseus* no 9, from the collection of the New Jersey Agricultural Experiment Station, and was subsequently found in fermentations with other strains, of different streptomycin-producing capacities, from the collection   Likewise, three ultraviolet mutants of *A   griseus*, morphologically distinct from the parent, were susceptible   Centraalbureau vor Schimmel-culture cultures labeled *A   griseus* Waksman and Curtis and *A   griseus* Bucherer were resistant to the action of the actinophage   However, no streptomycin was produced by these strains   Six additional species of *Actinomyces* were not affected by the actinophage

Effect of culture age   The actinophage multiplies at the expense of submerged cultures of *A griseus* of various ages   Complete lysis has been noted only with an inoculum consisting of spores of *A griseus*   Six hours after inoculation the cultures incubated with actinophage show a faint turbidity due to germinated spores   Shortly thereafter, the cultures lyse completely, and only occasion lly does a resistant colony grow out   With submerged vegetative inoculum, actinophage multiplication can be proved by the determination of numbers b the plaque method, but lysis is not complete   With 5 to 10 per cent by volume of submerged inoculum, there is little difference in degree of turbidity and streptomycin production in 24-hour-old control cultures and in cultures infected with actinophage   Usually, the infected cultures fragment at an earlier time than control cultures   Since streptomycin accumulation ceases about the time of fragmentation, yields are lower in infected cultures   The majority of *A griseus* cells which remain in the infected cultures following fragmentation are resistant

TABLE 2

Sensitivity of bacteriophages to chemicals in the absence of cells

AGENT*	VIRICIDAL DILUTION	
	E coli bacteriophage	*A griseus* acti o, 3
Acriflavine	<0 004 mg/ml	0 001 mg/ml
Actinomyces 34	1 250	1 32
Actinomyces 11	1 65	<1 2
Bacterium 24	1 250	<1 2

* Bacterial virus exposed to agent 16 hours at 37 C in nutrient broth substrate

to the action of the actinophage   Of 13 production lots of *A griseus* which fragmented early, 11 were found to contain actinophage

Actinophage resistant strains of A griseus   Several resistant cultures ha been selected following exposure of *A griseus* to the actinophage   Approx imately half of the isolates are equal to the parent in streptomycin production   Many appear to be lysogenic   One culture, which produced high yields of streptomycin in the presence or absence of added actinophage, always had two or three plaques of lysis in agar slant cultures   Filtrates of a series of four submerged culture transfers, in series, all contained approximately 100 plaq forming actinophage particles per ml for a sensitive strain of *A griseus*   The actinophage was capable of multiplying to a slight extent on the resistant isolate   Such cultures are dangerous for routine use in the production of strep tomycin, since conditions are favorable for the multiplication of any actino phage variants which gain the ability to attack the resistant culture

Sensitivity of actinophage to heat   The susceptibility of the actinophage heat was determined   Filtrates of a lysed culture of *A griseus* gro n in glucose "N-Z-amine" meat extract medium were used as a source of actinophage   No evidence of destruction occurred from heating a filtrate containing 2×

per cent of the actinophage particles survived, and at 90 C, 0 00002 per cent survived Only 0 5 per cent of 500,000,000 *A griseus* spores per ml of water remained viable after heating at 60 C for 15 minutes

Sensitivity of actinophage to chemicals Several compounds have been shown to destroy *Escherichia coli* bacteriophage during a 16-hour incubation at 24 C in nutrient broth, in the absence of bacterial cells Acriflavine, a filtrate from an unidentified bacterium, and filtrates from two actinomycetes have been most active The agents were tested against the actinophage under similar conditions The latter virus was more resistant than the *E coli* bacteriophage (table 2)

A griseus was inhibited by acriflavine in concentrations which were destructive to the actinophage The filtrate of *Actinomyces* 34, which had no

TABLE 3

Sensitivity of bacteriophages to chemical   n the presence of cells

AGENT	E coli PE IL		MG A griseus CULTURE†	
	Control	– Phage	Control	+ Phage
None	3×10^{11}	$2 < 10^3$	50	7
1 40 Act no 34	2×10^{11}	$3 10^8$	43	8
0 001 mg/ml Acriflavine	2×10^{11}	$5 < 10^6$	4	7
0 0001 mg/ml Acriflavine	1×10^{11}	2×10^3	1	3

  * Four hours' incubation
  † Twenty-four hours' incubation from spore inoculum 30 ml volume

inhibitory effect on growth of *A griseus*, did not retard lysis of *A griseus* by the actinophage (table 3)

Morphology of actinophage [2] Preparations made from cover slip impressions of plaques for electron microscope observation demonstrated the particulate nature of the lytic agent and its close resemblance to strains of *E coli* bacteriophage (Luria and Anderson, 1942) The chromium shadowing technique indicated a surprising diversity of structure of the actinophage particles (figures 2 and 3) Practically all particles had a long, relatively thick but bent tail of approximately 0 015 by 0 15 microns Whereas the majority of the heads appeared symmetrically spherical, 0 05 microns in diameter, many were composed of two distinct bodies and a few appeared to be similar to tetrads

One or two preparations had a majority of particles with two tails (figure 3) The heads did not appear sufficiently dense to indicate that these particles were simply overlying actinophages, and it seems possible that the preparations represented plaques formed by morphological variants of actinophage which
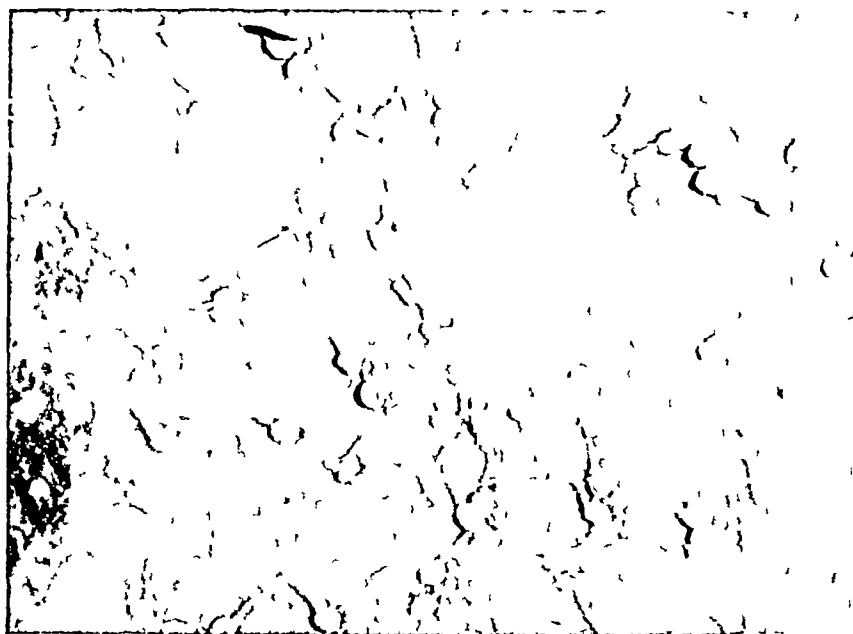
Fig 2 Electron Micrograph Showing the Actinophage   X 37 000
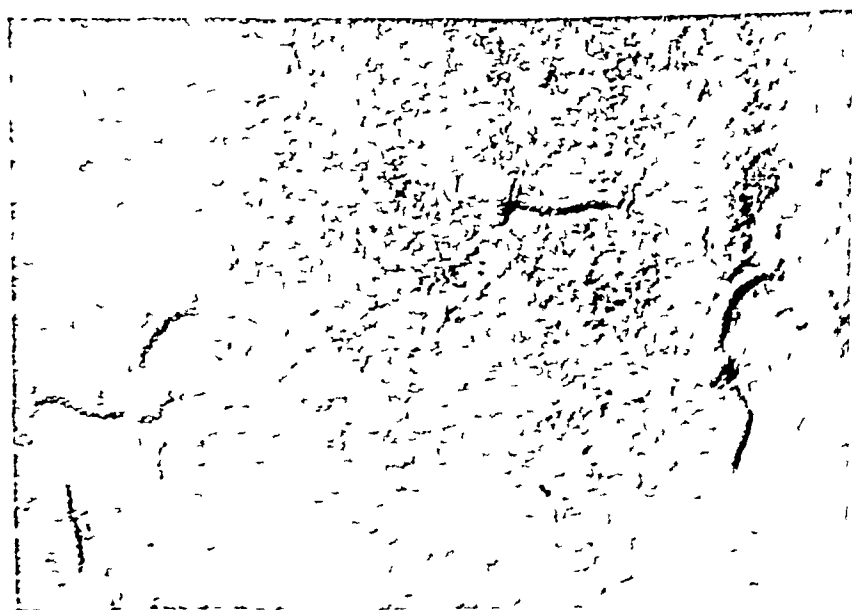
Fig 3 Electron Micrograph Showing Actinophage Particles Which Appear To Have Two Tails   × 95000

retained infectivity   However, no proof can be ᴜ.ᴇred at ᴘ ⌣ ' ᴜ ı⌣ origin of this unusual type   They were not present in the majority of the preparations

SUMMARY

An actinophage has been isolated which infects strains of *Actinomyces griseus* The virus is particulate, transmissible, and initiates lys s in young cells of *A griseus*   It is more resistant to heat than are the spores of *A griseus*, but is susceptible to certain viricidal agents which destroy *Escherichia coli* bacterio-phage   Resistant cultures of *A griseus* have been developed which may be lysogenic   Electron micrographs prove the particulate nature and demon-strate the morphology of the actinophage

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NOTE

THE RAPID RECOGNITION OF ASPERGILLIC ACID

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In examining unidentified microorganisms from natural sources in a search for new antibiotics, it is necessary to exclude previously known antibiotics This is usually done by running a so-called "bacterial spectrum" against a number of different test organisms which differ in sensitivity to the action of known antibiotic substances   However, even when the presence or absence of a known antibiotic is determined provisionally by this biological method, it is usually necessary to obtain additional evidence by chemical methods   This may require a large amount of extra work

An unidentified mold (our no 401) gave strong antibiotic activity (wide zones of inhibition of growth on agar plates) with both gram-positive and gram-negative bacteria   When cultured for 7 days or longer at 25 C, in 100-ml lots of trypticase soy broth (Baltimore Biological Laboratories) in 500-ml Erlenmeyer flasks, the surface became completely covered by a white mycelium, with areas of gray-green spores   The initial pH of 6 8 increased to 7 7 or higher Sterile filtrates neutralized to pH 7 also showed strong activity against bacteria Bacterial spectra run in comparison with a known sample of aspergillic acid strongly suggested that the latter acid was present   It was noted that *Pasteurella multocida*, Lederle strain, was inhibited by aspergillic acid in much higher dilutions than the other test organisms, making it a useful test organism in testing for this acid

In 1943 Menzel, Wintersteiner, and Rake (J Bact , **46**, 109) briefly mentioned that aspergillic acid is volatile with steam   This observation was applied by evaporating or distilling the alkaline broth cultures to about a third of the original volume   The concentrate was adjusted to pH 4 2 with HCl   Upon rapid distillation, a pale yellow, waxy-appearing, amorphous solid separated in the condenser and receiver   On standing overnight, more material separated in a microcrystalline form   The solid was dissolved out of the aqueous suspension, using chloroform in a separatory funnel   Evaporation of the extract in a current of air at room temperature yielded a viscous yellow residue which slowly hardened to a crystalline mass   The material was identified by means of its infrared spectrum, which agreed with that of aspergillic acid in all respects

Distillation of broth cultures adjusted to pH 4 2 without preliminary concentration gave a solution of aspergillic acid from which no solid separated However, the presence of the acid was demonstrated by adding 1 drop of reagent to 3-ml portions of the distillate   A strong brown color was obtained

with 1 N $FeCl_3$, 1 per cent copper sulfate gave a voluminous pale green precipi tate, and 1 per cent cobalt chloride gave a less abundant flesh-colored or pale orange precipitate or turbidity The saturated solutions remaining after solid aspergillic acid had separated from the distillates of preconcentrated cultures, and solutions of known aspergillic acid, gave similar reactions

These tests permit the recognition of aspergillic acid with a high degree of probability The tests proved useful with a second mold (our no 415) In this case it was possible to identify aspergillic acid with very little other work

It is also obvious that distillation may be used as a step in the preparation of aspergillic acid Distillation alone will not necessarily give a pure product, since several common fatty acids are appreciably volatile with steam It should also be noted that substances are known to exist which are very similar to aspergillic acid but not identical with it The volatility of these substances with steam is yet to be determined

PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MICHIGAN BRANCH

THE INCIDENCE OF ENTEROCOCCI IN HUMAN FECES *Morris F White, Joseph A Kasper, and Elizabeth J Cope*

Fecal specimens received in the laboratory of the Detroit Department of Health served as the source of material studied in this investigation The culture medium and methods as outlined by Winter and Sandholzer were adopted, however, the medium was modified to the extent that penicillin was omitted from the confirmation broth

In this series of cultures there was a total of 200 fecal specimens examined Positive presumptive findings were shown for all of the samples Of the total number, 115 showed the confirmatory findings for the presence of enterococci, but 85 cultures were considered negative Thus, organisms of the enterococcus group were recovered from 57 5 per cent of the cultures in this series

The repeated failure to isolate enterococci from the feces of 7 persons in this study indicates that some humans may not be carriers of enterococci at all times

These findings seemingly indicate that enterococci are not always present in the feces of humans Implicit reliance upon the finding of enterococci as a single indicator of human fecal pollution of water cannot, as yet, be accepted without question

AN ACTINOPHAGE IN STREPTOMYCIN-PRODUCING CULTURES OF STREPTOMYCES GRISEUS *R M Smith, W H Kuhn, and G R M Miesel*

An actinophage which affects cells of *Streptomyces griseus* has been found Its presence has been noted in stock cultures and in fermentation beers of various types Plaques typical of bacteriophage action were found when infected cultures were grown on agar media and lysis was noted in cultures grown by submerged methods The lytic agent increases in quantity upon cultivation of the infected cultures, passes through Seitz and other bacteriological filters, and is relatively heat stable

Examination of stock cultures revealed that most of them were infected, and attempts were made to render cultures phage-resistant Exposure of the susceptible cultures to the phage under various conditions resulted in the development of resistant strains These strains, thus far, have shown no tendency to revert to susceptibility The streptomycin-producing capacity of the strains which we have rendered resistant has not differed appreciably from that of the parent cultures

NORTHERN CALIFORNIA-HAWAIIAN BRANCH

SELECTIVE BLOOD FACTORS AFFECTING BACTERIAL VARIATION *Werner Braun,* Division of Veterinary Science, University of California, Berkeley, California

The selective factor suppressing the establishment of nonsmooth variants of *Brucella abortus*, previously demonstrated in normal serum of various *Brucella* susceptible animals, has been found in the gamma globulin fraction *In vivo*, modifications of the gamma globulin, which occur after vaccination, alter the selective activity of normal gamma globulin *In vitro*, preliminary tests have indicated that the selective activity of normal gamma globulin disappears in the presence of sufficient anti gamma-globulin (produced by inoculation of bovine gamma globulin into rabbits) Similarly, in the presence of high albumin concentrations, corresponding to approximately twice

the normal blood concentrations, gamma globulin fails to express its selective activity   It is hoped that this information will lead to the creation of *in vivo* conditions which will favor the establishment of non-smooth, avirulent variants

PENICILLIN STABILITY IN PHOSPHATE, ACETATE, AND CITRATE BUFFERS   *John O Thomas*, Biological Research Department, Cutter Laboratories, Berkeley, California

The stability of crystalline potassium penicillin G (1,530 units per mg) in NaH_2PO_4-Na_2HPO_4 buffers (pH 6 0), of final molarities M/16, M/50, M/100, and M/200, and in M/50 acetate and M/50 citrate buffers was studied for a maximum of 86 days, the initial potencies of the sterile mixtures being approximately 10,000 units per ml   Sealed 5-ml volumes of each mixture were kept at 37, 24, and 2 C, one set of mixtures in a temperature group being cup-assayed against *Staphylococcus aureus* (NRRL 318), and the pH's being measured, on a particular day   Residual activities were computed as percentages of zero time potencies

Penicillin destruction at 37 C was rapid, first-order curves resulting   Similar less steep curves were encountered at 24 C   At both temperatures protection efficiency followed buffer capacity, with the exception of citrate, which was the most efficient

At 2 C, a first order inactivation curve resulted for the saline control   The buffered mixtures' curves, however, all showed periods, from 10 days (M/100 phosphate) to 72 days (acetate), when the activities did not drop below 100 per cent   These indicate activity potentiation because maximal potencies, for example, of 150 per cent and 138 per cent (assay error about 10 per cent) occurred in the acetate and M/200 phosphate buffers, respectively, and these in spite of corresponding pH drops to 5 50 and 5 30

Except for saline and M/200 phosphate, all 2 C curves showed an initial rise, a moderate fall, and a second rise before final drops, the rises being independent of pH drops, though pH's remained practically constant in citrate and M/16 phosphate   No second rise occurred in M/200 phosphate, the pH of which (4 70) was the lowest of the buffers, at 50 days   Acetate provided the best protec-

tion, despite a pH fall to 5 20 at 86 days   The buffer ions are apparently concerned with these phenomena

AN IMPROVED TECHNIQUE FOR BACTERIOLOGICAL CULTURE STUDIES   *Phillip J Brady and Paul Esau*, Research Laboratories, California Packing Corporation, San Francisco, California

A simple, convenient, and inexpensive double compartment culture tube for fermentations and aerobic and anaerobic culture studies has been designed   Its uses can be enumerated as follows

(1) A liquid medium or agar is put in the long arm and pyrogallol in the short arm of the tube   Anaerobes can be cultured by closing the tube with a rubber stopper after a cotton plug

(2) The nature of a gas produced by bacteria (usually CO) can be detected by putting lime water (filtered) in the short arm   Precipitation of calcium carbonate designates CO_2

(3) Partial neutralization of acid media by hydrolysis during sterilization is avoided by placing the neutral medium in the long arm and the acid medium in the short arm and mixing together after autoclaving and cooling

(4) Carbohydrate media for fermentation studies can be prepared by placing the sugar solution in the short arm, and the peptone broth with indicator and gas vial in the long arm   After being autoclaved for 10 to 15 minutes at 10 to 15 pounds' pressure, the medium is cooled and the ingredients combined in the long arm   The short arm can now be used for the detection of gas or for creating anaerobic conditions

DECOMPOSITION OF TARTRATES BY SOME MESOPHILIC, SPOREFORMING, OBLIGATE ANAEROBES   *Joseph Tabachnick*, Division of Food Technology, University of California, Berkeley, California

Since the classical experiment performed by Pasteur in 1863 in which he demonstrated the existence of obligate anaerobes (with calcium tartrate as a substrate), very little work has been done with the obligatory anaerobic bacteria which decompose tartrate   None of the later investigations were made with pure cultures

Twenty three strains of tartrate fermenting clostridia were isolated by an enrichment technique from calcium tartrate recovery equipment and spoiled calcium tartrate, as well as from soils

With the exception of their ability to utilize glycerol and tartrate, the majority of the strains isolated were closely related to the type species, *Clostridium butyricum*, as described in Bergey *et al* (1939) Glucose was fermented with the production of carbon dioxide, hydrogen, butyric and acetic acids, and very small amounts of neutral volatile products Tartrate was fermented with the production of drogen, and acetic acid, and small amounts of butyric acid and ethanol Trace amounts of pyruvic acid from the tartrate fermentation were isolated and identified

With the exception of *l*-malic acid, four carbon dicarboxylic acids other than *d*-tartaric acid were not attacked by the cultures investigated

The enzymes involved in the decomposition of tartrate were shown to be adaptive in character Attempts to adapt other cultures of the common saccharolytic clostridia to the utilization of tartrate were unsuccessful

STUDIES ON POLYMYXIN ISOLATION AND IDENTIFICATION OF BACILLUS POLYMYXA AND DIFFERENTIATION OF POLYMYXIN FROM CERTAIN KNOWN ANTIBIOTICS

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Polymyxin is an antibiotic substance occurring in the culture filtrates of *Bacillus polymyxa*   The isolated substance is unique in its specificity for gram-negative bacteria   A summary of the more important results obtained during the course of several years, including chemotherapeutic and toxicity data, has been reported (Stansly, Shepherd, and White, 1947)   The present contribution is concerned with the isolation and identification of the antibiotic-producing organism and some early findings which both characterized and distinguished polymyxin from certain known antibiotics

Isolation of Bacillus polymyxa.   *Bacillus polymyxa* was isolated from soil in the course of a program designed to find new antibiotics for the chemotherapy of gram-negative bacterial infections   The test organism used in this search was *Salmonella schottmuelleri*   Our method for isolating antibiotic-producing organisms with a specific type of activity involves the preparation of pour plates of soil dilutions using a variety of media and cultural conditions   The plates are subsequently sprayed with a suspension of the test organism by means of an apparatus designed for the purpose (Stansly, 1947)

Identification of Bacillus polymyxa   The identification of *Bacillus polymyxa* was established by following the key to the identification of aerobic sporeforming bacteria by Smith, Gordon, and Clark (1946)   In the preliminary work,[1] edition 5 of Bergey's *Manual of Determinative Bacteriology* (1939) and the galley proofs of edition 6 were found helpful

An 18-hour broth culture consisted of gram-negative rods with few or no gram-positive cells   Older cultures showed vegetative cells and oval spores either free or central to terminal in adhering and swollen sporangia   Broth cultures at 30 C were turbid and had a ropy sediment   Indole was not formed   Nitrates were reduced to nitrites   Hydrogen sulfide was not produced   Acid and gas were formed from glucose, lactose, and sucrose   Acid but no gas was produced from rhamnose and a slight amount of acid but no gas from sorbitol   Starch was hydrolyzed   Acid and gas were produced from litmus milk, which was coagulated and reduced

The existence of oval spores, central to terminal, and sporangia frequently adhering and swollen, plus the predominant gram-negative nature of the vegeta-

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[1] The authors are indebted to Dr Walter C Tobie and Miss Marion H Cook for the preliminary work which led to the conclusion that the antibiotic-producing organism had characteristics intermediate between those of *Bacillus polymyxa* and *Bacillus macerans*

tive forms, placed the organism in group 2 in the classification of Smith, Gordon, and Clark   The fermentation of carbohydrates, such as glucose, lactose, and sucrose, with the formation of both acid and gas narrowed the possible identity of the organism to one of two species, namely, *Bacillus polymyxa* or *Bacillus macerans*   These two species may be distinguished in the following ways (1) *B polymyxa* produces acetylmethylcarbinol from the proper substrate, whereas *B macerans* does not, (2) *B polymyxa* does not produce an amylase which catalyzes the formation of crystalline dextrins from starch, whereas *B macerans* produces this enzyme   Both of these criteria were used to identify the unknown organism [2]

Production of acetylmethylcarbinol   Three known *B polymyxa* strains (ATCC nos 8523, 7047, and 7070), one *B macerans* (ATCC no 355), and the unidentified organism were inoculated in the recommended neopeptone medium and under the suggested conditions (Smith *et al*, 1946)   The test for acetylmethyl carbinol was made according to O'Meara (1931)   *B macerans* was negative for acetylmethylcarbinol on the third, fifth, seventh, and fourteenth day of incubation, whereas the isolated organism and the three polymyxa strains were positive at these times

Formation of crystalline dextrins   The formation of crystalline dextrins from starch was detected by the iodine test of Tilden and Hudson (1942)   The same strains of *B polymyxa* and *B macerans* were used as before, in a medium and under conditions recommended (Smith *et al*, 1946), with the exception that Merck's soluble starch was used instead of Takamine or White Rose   *B macerans* gave a positive test for crystalline dextrins (both hexagons and needles were observed) when tested after 2 weeks and again after 3 weeks of incubation All three polymyxa strains and the antibiotic-producing organism were negative at these times

The two foregoing critical tests supported each other in identifying the organism as a strain of *Bacillus polymyxa*, a species apparently first described in 1880 as *Clostridium polymyxa* (Smith *et al*, 1946) and of current interest in the production of 2,3-butanediol by fermentation (Adams, 1946)

Antibacterial activity   When a colony of *Bacillus polymyxa* on an agar plate was sprayed with a suspension of *Salmonella schottmuelleri* or *Escherichia coli* and compared to a similar plate sprayed with *Staphylococcus aureus*, the difference in the inhibition zones of the gram-negative and the gram-positive organisms was striking, the former showing a wide zone (approximately 40 mm), the latter a relatively narrow zone (approximately 10 mm)   It was this difference alone which stimulated further investigation since, at the time the investigation began, no antibiotic had been described which was more active against gram-negative bacteria than gram-positive bacteria

At first some difficulty was experienced in demonstrating antibacterial activity in bacteria-free broth filtrates   This may have been due to the use of filters which removed the active principle   With the introduction of sintered

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[2] The authors wish to thank Miss Nydia H Ananenko for conducting these two tests in the identification of *Bacillus polymyxa*

_ _ rum c am   w t filtered broth is given in table 1 [3]

As shown in table 1, crude fermentation liquor was highly active against the gram-negative bacteria but either inactive or relatively inactive against the gram-positive organisms, confirming and extending the previous findings with the *Bacillus polymyxa* colony   More striking than the results with crude fermentation liquor were those obtained with concentrates of polymyxin These were relatively free of activity against gram-positive bacteria, even against those organisms, for example, *Diplococcus pneumoniae* SVI, which were some-

TABLE 1

Antibacterial spectrum of polymyxin broth filtrates*

ORGANISM	MEDIUM†	HIGHEST INHIBITORY DILUTION‡
Escherichia coli	A, 1/16	1,024
Eberthella typhosa	A, 1/16	2,048
Shigella dysenteriae (Flexner)	A	512
Salmonella schottmuelleri	A, 1/16	128
Pseudomonas aeruginosa	A, 1/16	128
Klebsiella pneumoniae	A, 1/16	512
Streptococcus, group A, strain C203	A	8
Streptococcus, group B	A, 1/4	4
Streptococcus, group D	A, 1/2	0
Diplococcus pneumoniae, type I	A	32
Staphylococcus aureus	A, 1/16	0
Clostridium welchii	B	16
Erysipelothrix rhusiopathiae	B+	8

* The medium consisted of glucose, glycerol, tryptone, yeast extract, and inorganic salts, and was therefore far more complex than the routine production medium which was finally developed (Stansly *et al* , 1947)

† A = Trypticase-soy-phosphate broth (Baltimore Biol Lab)   A, 1/2, 1/4, and 1/16, designates the medium used at 1/2, 1/4, and 1/16 the concentration recommended by the manufacturer

B = Brewer's thioglycolate broth

B+ = Brewer's thioglycolate broth + bile and yeast extract

‡ Inhibitory end point obtained by serial twofold broth dilution

what affected by the crude liquor   Thus from table 1 it can be calculated that *E coli* is 32 times more sensitive to the broth filtrate than is *D pneumoniae*   With a partially purified preparation of polymyxin the ratio was found to be in excess of 2,048 [4]

A possible explanation for the difference in behavior of the liquor and concentrates was that the liquor contained at least two active substances, only one of which, the gram-negative principle, was present in the concentrates   In

support of this explanation is the fact that, as described below, it has been possible to extract from the cells of *Bacillus polymyxa* a water-insoluble, ethanol soluble substance which is highly active against *Staphylococcus aureus* and in active against *E coli*   It is suggested, therefore, that the low order of activity of metabolic liquors against gram-positive bacteria may be due to small amounts of this cellular substance escaping into the medium

Ten grams of moist, unwashed *Bacillus polymyxa* cells and cellular debris, collected by centrifugation, were triturated with sand to a smooth paste   Fifty ml of 95 per cent ethanol were added and the suspension was shaken overnight at room temperature   To 40 ml of the alcoholic filtrate, 80 ml of water were added and the resulting precipitate was collected and dried   It was then dissolved in boiling 95 per cent ethanol and treated several times with charcoal to decolorize it   Water was added to the point of incipient turbidity and the solution cooled   The flocculent white precipitate was washed with ethanol and ether, and dried   A 200-mg per cent suspension was made in water and tested for activity against *E coli* (MacLeod) and *S aureus* (Barlow) by the agar streak method   The suspension inhibited *S aureus* at 10 μg per ml and was inactive against *E coli* at 1,000 μg per ml   The origin (Stokes *et al*, 1942), solubility properties (insoluble in water, ether, chloroform, and acetone), and biological behavior are similar to those of tyrothricin, although its relationship to tyrothricin has not otherwise been determined

Effect of blood on activity   Before therapeutic experiments were instituted it was felt desirable to determine the effect of blood on the antibacterial activity of polymyxin and to determine whether the active substance contained any hemolytic principle   On blood agar plates a colony of *Bacillus polymyxa* showed a very narrow but distinct zone of hemolysis   However, the antibacterial zone (*E coli*) was much greater

The experiment summarized in table 2 demonstrated that blood had no appreciable effect on the antibacterial activity of polymyxin, nor had polymyxin, in the concentrations used, any visible effect on blood   Also, the fact that the last tubes showing no growth apparently contained no viable cells suggested that polymyxin had bactericidal properties

Differentiation of Polymyxin from Known Antibiotics

Polymyxin is active only against certain gram-negative bacteria (Stansly *et al*, 1947)   This fact alone would distinguish it from all known antibiotics It may be worth while, however, to point out these and other differences insofar as the literature or actual comparisons in the laboratory permit

Tyrothricin   The insolubility of tyrothricin and its components in water (Hotchkiss and Dubos, 1941, Dubos and Hotchkiss, 1942), its hemolytic activity (Dubos and Hotchkiss, 1942), its toxicity (Robinson and Molitor, 1942), and its greater activity for gram-positive compared to gram-negative organisms (Dubos and Hotchkiss, 1941) distinguished it from polymyxin

Streptomycin and streptothricin   Both streptomycin and streptothricin have gram-positive activity, thus distinguishing them from polymyxin   Neverthe-

... certain other respects was notable These were their basic nature (Waksman, Bugie, and Schatz, 1944), water solubility (Waksman and Schatz, 1945), high activity against certain gram-negative bacteria (Waksman *et al*, 1944), similarity in concentration procedure (Waksman and Schatz, 1945, Stansly *et al*, 1947), and high activity of streptomycin in the *Klebsiella pneumoniae* mouse infection (Heilman, 1945) In view of these similarities, it was felt desirable to compare polymyxin, streptomycin, and streptothricin experimentally to determine whether any close relationships existed among them

The effect of the pH of the medium on the antibacterial activity of streptomycin and streptothricin is well known (Foster and Woodruff, 1943, Waksman

TABLE 2

Effect of blood on the antibacterial activity of polymyxin and the effect of polymyxin on blood*

CONC. POLYMYXIN MG PER CENT	50 PER CENT BLOOD		20 PER CENT BLOOD		10 PER CENT BLOOD		NO BLOOD
	Growth	Hemolysis	Growth	Hemolysis	Growth	Hemolysis	Growth
32	−	−	−	−	−	−	−
1/8	−	−	−	−	−	−	−
1/16	−	−	−	−	−	−	−
1/32	−†	−	−	−	−	−	−
1/64	+		−†		−†		−†
1/128	+		+		+		−
1/256	+		+		+		+

* Serial twofold dilutions of a crude polymyxin concentrate were made in trypticase-soy-phosphate broth containing the indicated concentrations of defibrinated rabbit blood Each tube contained a total of 2 ml and was inoculated with approximately 700 *E coli* cells Incubation was for 24 hours at 37 C, and the presence or absence of growth was determined by visual inspection This was possible since the red blood cells had settled by this time

† These tubes were plated out on agar (1 ml from the tube + 13 ml agar) and incubated for 48 hours at 37 C No visible colonies appeared on any of the plates

and Schatz, 1945) and seemed a plausible basis for comparison Another, obviously, was an antibacterial spectrum with selected organisms The results of tests using these criteria are shown in tables 3 and 4

The anticipated increase in activity with increasing pH in the case of streptomycin and streptothricin (table 3) was confirmed, whereas polymyxin showed essentially no change in activity under the same circumstances The data show that, under the conditions employed, streptomycin was 16 times more active at pH 8 5 than at pH 5 5 and streptothricin 78 times more active at pH 8 5 than at pH 5 5

The data in table 4 indicate that the preparation of polymyxin used in this experiment was 16 times more active against *E coli* than was streptomycin, but less than one sixteenth as active as streptomycin against *Bacillus mycoides* Likewise, the preparation of streptothricin was twice as active as polymyxin against *E coli* but over 80 times as active against *Bacillus subtilis* These ob-

servations comprised presumptive evidence for the nonidentity of polymyxin
with streptomycin or streptothricin   Cross-resistance experiments with poly
myxin and streptomycin confirmed this presumption (White and Clapp, to be
published)   Additional biological and chemical properties which distinguish
polymyxin from streptomycin and streptothricin have been found and will be
reported elsewhere

  Subtilin   The relative insolubility of subtilin in water at neutrality (anony
mous, 1946) and its inactivity against most gram-negative bacteria (Salle and
Jann, 1945) distinguished subtilin from polymyxin   The susceptibility of

TABLE 3

Effect of pH of assay medium on the inhibition of E coli

EXPERIMENT	ANTIBIOTIC†	CONC IN MG PER CENT INHIBITING GROWTH OF E COLI* AT INITIAL pH VALUES OF			
		5 5	6 5	7.5	8.5
1	Polymyxin	0 19	0 39	0 39	0 39
	Streptomycin	25 0	25 0	1 56	1 56
2	Polymyxin	0 19	0 09	0 09	0 09
	Streptothricin	1 56	0 39	0 09	0 02

* In T-S-P medium, agar streak method
† Antibiotic solutions adjusted to pH 6 4 and titrated in media of indicated pH

TABLE 4

Relative antibacterial activity of polymyxin, streptomycin, and streptothricin

EXPERIMENT	ANTIBIOTIC	MINIMUM EFFECTIVE CONC * MG PER CENT		
		E coli	B mycoides	B subtilis
1	Polymyxin	0 5	>32	
	Streptomycin	8 0	2	
2	Polymyxin	0 09		>2,000
	Streptothricin	0 04		25

* In T-S-P medium, agar streak method

subtilin to decomposition by pepsin, trypsin, and pancreatin (anonymous, 1946)
and the resistance of polymyxin to these enzymes (Stansly and Ananenko, to be
published) confirmed the lack of identity

  Bacitracin (Johnson, Anker, and Meleney, 1945)   Its activity against gram
positive bacteria and lack of activity against gram-negative bacteria were the
only criteria available which served to distinguish bacitracin from polymyxin

  Eumycin (Johnson and Burdon, 1946)   The solubility of eumycin in acetone
and its inactivity against *Eberthella typhosa* and *E coli* distinguished it from
polymyxin

  Gramicidin S   Its insolubility in water (Belozersky and Passhina, 1944),

Pitskhelauri, 1944), and greater or equivalent activity against gram-positive organisms compared to gram-negative organisms (Gause and Brazhnikova, 1944) distinguished this substance from polymyxin

Colistatin (Gause, 1946)   Its higher activity against staphylococci than against *E coli* and its inextractability from broth filtrates with normal butanol were characteristics distinguishing this recently described material from polymyxin

Bacillin (Foster and Woodruff, 1945)   Bacillin is equally effective against gram-positive and gram-negative bacteria   Blood neutralizes its activity *in vitro*   These facts distinguished bacillin from polymyxin

Antibiotic from Bacillus licheniformis (Callow and Hart, 1946)   Its greater activity against *S aureus* than *E coli* , activity against *Mycobacterium tuberculosis*, and apparent insolubility in ethanol distinguished this recently described material from polymyxin

SUMMARY

The isolation and identification of *Bacillus polymyxa* as the organism producing the antibiotic polymyxin is described   Preliminary data on the biological activity of polymyxin which served both to distinguish and characterize the antibiotic are given   The points of distinction between polymyxin and some known antibiotics which bore a superficial resemblance to polymyxin are discussed

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MICROBIOLOGICAL AGENCIES IN THE DEGRADATION
OF STEROIDS

II Steroid Utilization by the Microflora of Soils

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An investigation of the degradation of cholesterol by soil microorganisms has shown that the initial oxidation of this compound is due, both *in situ* and in pure culture, to the activities of members of a single genus, *Proactinomyces* (Turfitt, 1944a) During the cultural work a considerable number of organisms, both molds and bacteria, persisted with the strains of *Proactinomyces* through many subcultures, but when isolated and inoculated into synthetic medium with cholesterol as sole C source, were unable to survive Since *Proactinomyces* oxidation results in the formation of Δ^4-cholestenone, and subsequently in actual molecular fission (Turfitt, 1944b, 1947), it may well be that the further products resulting from *Proactinomyces* oxidation constituted a substrate for the growth of the attendant organisms

In the case of steroid compounds lacking the intact hydrocarbon C_{17} side chain, oxidations at —OH groups giving the corresponding keto compounds have been reported with several groups of organisms, *Flavobacterium dehydrogenans* (Arnaudi, 1942), *Flavobacterium androstenedionicum* (Ercoli and Molina, 1944), *Flavobacterium carbonilicum* (Molina and Ercoli, 1944), *Alcaligenes faecalis* (Schmidt, Hughes, Green, and Cooper, 1942, Hughes and Schmidt, 1942), *Escherichia coli* (Schmidt and Hughes, 1944), and pseudodiphtheria bacilli (Zimmermann and May, 1944) These investigations, however, have been concerned essentially with ketone formation, and, in general, alternative carbon sources such as serum or yeast water have been included in the cultures with the object of obtaining a vigorous multiplication of the bacterial cells and a consequent high dehydrogenase concentration Synthetic media with cholic acid as the sole carbon source have been used by Schmidt, Hughes, Green, and Cooper (1942) in oxidations with *Alcaligenes faecalis*, and triketocholanic acid has been isolated as the end product of the bacterial action In this instance it is clear that the carbon available for growth has been derived from a breakdown of the bile acid molecule

The only general restrictive influence on bacterial utilization thus far reported is due to the side chain, and the available evidence suggests that the modified natural sterol skeleton is probably susceptible to attack by a variety of organisms

EXPERIMENTAL RESULTS

Although the basic ring structure of all steroid compounds is fundamentally the same, the variety of compounds resulting from the introduction of various

substituents is extremely wide and is in addition vastly increased by the complex stereoisomeric configurations in the steroid rings   It was clearly impracticable to investigate more than a limited selection of compounds, and a choice was made with a view to determining the influence on the microbiological utilization of (1) the length and nature of the side chain, (2) the presence of free and esterified —OH groups, (3) the presence of ketonic groups in the more usual C_3 and C_{17} positions, (4) the presence of free or "blocked" double bonds, (5) the cis- and trans-decalin configurations of rings A and B, (6) stereoisomerism at C_5, and (7) the presence of benzenoid rings in place of the complete saturation of the polyhydrocyclopentanophenanthrene skeleton

Isolation technique   The process of selective isolation used in the investigation of the cholesterol-decomposing organisms of soils (Turfitt, 1944a) was again adopted, although certain modifications were considered desirable owing to the varied physical and chemical properties of the steroid substrates

The soil samples were restricted to the British Isles, and the 20 specimens taken constituted a range covering both virgin and cultivated ground   In each instance the sample was collected from immediately below the soil surface in a sterile 4- by 1-inch tube   No attempt was made to investigate the microflora present at a greater depth

Sterilization of the individual steroid materials to be used in the cultures presented a somewhat difficult problem   In the case of cholesterol, steam sterilization was entirely effective, but this method was obviously unsuitable with other steroids Δ^4-Cholestenone, for example, has a melting point of 80 C and on cooling separates as a hard, solid mass   Again, there can be no guarantee that in the case of some of the less stable steroids heat treatment does not result in a slight decomposition yielding a material containing traces of impurity   The same considerations hold with regard to sterilization by ultraviolet light, since with ergosterol, and probably also in minor degree with certain other steroids, molecular transformation results   A successful solution of the problem was achieved by recrystallization of the compounds from suitable solvents, with filtration and drying conducted under aseptic conditions   The materials were stored in sterile tubes and were tested for sterility before use by streaking on both nutrient agar and wort agar plates

Bacteria and actinomycetes   Conical culture flasks (100-ml), each containing 30 ml mineral salt solution (NH_4NO_3, 0 1 per cent, K_2HPO_4, 0 025 per cent, $MgSO_4$ $7H_2O$, 0 025 per cent, NaCl, 0 0005 per cent, $FeSO_4$ $7H_2O$, 0 00001 per cent) were autoclaved at 115 C for 10 minutes, and approximately 1 mg steroid was introduced aseptically

Molds   In devising a method to ensure a normal surface development of mold mycelium, a distinction was drawn between the slightly soluble carboxylic acids, cholic acid, and 3-hydroxy-Δ^5-cholenic acid, and the insoluble steroid compounds   For the former, 100-ml Gates' culture flasks, each containing 50 ml of mineral salt solution ($NaNO_3$, 0 2 per cent, KH_2PO_4, 0 1 per cent, $MgSO_4$ $7H_2O$, 0 05 per cent, KCl, 0 05 per cent, $FeSO_4$ $7H_2O$, 0 01 per cent), were sterilized by steaming for 1 hour on each of 3 successive days, and ap-

compor · petri dishes were c · oyed , each contained a ·uic , compact layer of glass wool previously purified by acid and alcohol treatment and just covered with the mineral salt solution The dishes were sterilized by steaming, and approximately 1 mg steroid was sprinkled with aseptic precautions over the surface

Approximately 0 5 ml of a heavy aqueous suspension of each of the 20 soil samples were transferred to each of the flasks and dishes containing the various steroid compounds, and the cultures were incubated aerobically at 25 C   After 7 days a loopful from each vessel was transferred to a duplicate containing fresh medium, this procedure being repeated three times From the final cultures transfers were made on (a) nutrient agar, (b) casein agar, and (c) Czapek-Dox agar   The organisms which appeared on these plates were isolated and incubated in pure culture with the various steroid-containing media   Increased bacterial count or development of mold mycelium, together with alteration of the pH of the medium, was regarded as evidence of steroid utilization

Description and distribution of isolated organisms   In this investigation of the aerobic organisms of soils, 20 soil samples and 20 steroid compounds, under two separate cultural conditions, involved 800 initial cultures   After the subsequent "purification" cultures, numbers of organisms were isolated which failed to survive on the appropriate pure steroid No mention is made of these organisms in table 1, which summarizes the numbers and general types of steroid-decomposing organisms isolated from particular classes of soils   Strains of *Proactinomyces* are indicated by "P," and the numbers of strains isolated are given in parentheses   Gram-negative rods are indicated by "gm- "

Description of isolated strains   In this survey, 313 of the 355 cultures of bacteria isolated consisted of gram-positive rods, or of long or short filaments breaking up in older culture into short rods or coccoid forms   The organisms have been cultured on a wide variety of media, and in the majority of cultures, especially upon the less rich media, aerial mycelium was produced in greater or less degree, in no instance was there evidence of spore formation upon examination by the method of Orskov (1923)   In cultural and morphological characteristics the organisms fall essentially within the genus *Proactinomyces* and for the most part have the softness and translucency of the α-type of colony (Umbreit, 1939)   In several cases the strains did not show a strict agreement with the characteristics of known types, but the divergencies were insufficient to justify new species rank, and they have been regarded rather as variants of existing species   The divergencies were particularly marked in respect to acid-fastness, a character which was found to be influenced markedly by the composition of the culture medium   This feature of *Proactinomyces* has previously been reported by Jensen (1931, 1932) in a detailed taxonomic study of the genus

The 298 cultures of this group have thus been classified as follows *P opacus* (135 cultures), *P erythropolis* (126 cultures), *P globerulus* (17 cultures), *P coeliacus* (8 cultures), *P aquosus* (5 cultures), *P crystallophagus* (5 cultures), and *P agrestis* (2 cultures)

TABLE 1

Steroid-decomposing organisms from varied soil types

STEROID	SOIL TYPES (4 SAMPLES EACH TYPE)				
	Acid Sand	Loam	Marl	Alkaline Peat	Arable
Stigmasterol	P (3)	P (4)	P (4)	P (2)	P (6)
β-Sitosterol	P (4)	P (4)	P (5)	P (3)	P (4)
Ergosterol	P (3)	P (5)	P (3)	P (3) gm (1)	P (6) gm (2)
Coprosterol	P (3)	P (3)	P (3)	P (2)	P (5)
Dihydrocholesterol	P (4)	P (4)	P (4)	P (3)	P (7)
epi-Dihydrocholesterol	P (3)	P (3)	P (4)	P (3)	P (6)
Cholesterol acetate	P (3)	P (4)	P (3)	P (2)	P (5)
Coprosterol acetate	P (3)	P (4)	P (4)	P (3)	P (6)
Cholesteryl chloride	None	None	None	None	None
Cholesterol acetate dibromide	None	None	None	None	None
Dicholesteryl ether	P (4)	P (4)	P (4)	P (2)	P (4)
Δ^4-Cholestenone	P (4)	P (5)	P (4)	P (3)	P (6)
Coprostanone	P (3)	P (3)	P (4)	P (3)	P (5)
Androsterone	P (4) gm (2)	P (5) gm (1)	P (5)	P (3)	P (6) gm (1)
trans-Dehydro-androsterone	P (4) gm (3)	P (4) gm (2)	P (4) gm (1)	P (2) gm (1)	P (6) gm (3)
Progesterone	P (3) gm (1)	P (3) gm (1)	P (3) gm (1)	P (3)	P (4) gm (2)
3-Hydroxy-Δ^5-cholenic acid	P (4) gm (3) molds (1)	P (5) gm (2)	P (4) gm (1)	P (3) gm (1)	P (5) gm (2) molds (1)
Cholic acid	P (4) gm (5) molds (2)	P (4) gm (3) molds (1)	P (3) gm (1)	P (3)	P (4) gm (2) molds (1)
α-Oestradiol	P (1)	None	None	None	P (2)
Oestrone	None	None	None	None	P (1)

, addition, two cultures have been provisionally identified as *Mycobacterium phlei* An authentic strain of *M phlei* has previously (Turfitt, 1944a) been found unable to utilize cholesterol, and in consequence tests for cholesterol-decomposing ability have been conducted with a number of standard strains of this organism The property, which was fairly vigorous in some strains, was entirely lacking in the majority and could not, furthermore, be stimulated by enrichment cultures Closely comparable findings have been experienced with cultures of *Mycobacterium smegmatis* and *Mycobacterium stercoris*

The β-group of *Proactinomyces* was represented by 13 cultures which have been thus identified *P asteroides* (8 strains), *P farcinicus* (4 strains), and *P paraffinae* (1 strain)

In addition to the *Proactinomyces*, 42 cultures of gram-negative bacteria were obtained Of these, 29 were short rods with 1 to 5 polar flagella and on asparagine agar (Georgia and Poe, 1931) developed the green fluorescence of *Pseudomonas* The blue pigment pyocyanin, typical of *Pseudomonas aeruginosa*, was not detected even in glycerol peptone agar (Gessard, 1891, Turfitt, 1936) Neither these nor the 13 cultures of gram-negative nonfluorescent organisms have as yet been satisfactorily classified, but they are being incorporated in a further study of steroid utilization specifically by gram-negative organisms Ercoli (1938), in the attempted bacterial reduction of male sex hormones to etiocholane derivations, has described the culture of *Pseudomonas fluorescens*, and also of *Escherichia coli*, in meat broth in the presence of 200 mg androstenedione No hydrogenation products could be isolated, but 86 mg of unchanged dione were recovered Similar results were obtained with *trans*-dehydroandrosterone This apparent utilization of the steroid by these organisms is thus in accord with the present results indicating that in such modified steroids gram-negative bacteria play a not inappreciable part

The only instances in which isolated molds were able to survive repeated transfer in pure culture were with the free acids, cholic acid, and 3-hydroxy-Δ^5-cholenic acid In all, six molds (identified as species of *Penicillium*, *Aspergillus*, and *Alternaria*) were found to yield a few straggling hyphae on the surface of the medium with the petri dish, glass wool technique A definite mycelial felt never developed There was no change in the pH of the medium, and no detectable ketone formation Steroid decomposition by these organisms was clearly of a negligible order, and they were accordingly not further investigated

SUMMARY AND CONCLUSIONS

Cultural conditions are described by which the microflora of soils have been tested for ability to utilize a variety of steroid materials

Steroids generally, with a very few special exceptions such as halogen substi
tuted derivatives, are attacked by *Proactinomyces* of soils, and these are clearly
the predominant organisms in steroid decomposition

With steroid compounds in which the C_{17} side chain is modified or lacking,
certain gram-negative bacteria, expecially of the fluorescent type, can utilize the
molecule

Although species of *Penicillium*, *Aspergillus*, and *Altenaria* have been found to
survive repeated transfer in pure culture on soluble carboxylic acid derivatives,
the paucity of the growth and the lack of evidence of steroid decomposition are
taken to indicate that these fungi are of small significance in the utilization of
steroids in nature

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ACTINOMYCETES AGAINST VIRULENT HUMAN TYPE
TUBERCLE BACILLI[1]

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A simple way to test the antibiotic properties of an organism is to streak it on an agar plate and then, after growth has been established, cross-streak with the organism against which it is to be tested (Waksman, 1945)   The zone of inhibition of the test organism can then be measured   Thus, by cross-streaking with many organisms, it becomes fairly easy to establish a "spectrum" of the inhibiting properties of any bacterium, mold, or actinomycete which will grow discretely on an agar plate

Once the bacteriostatic properties of an agent have been established, there are many ways of testing it quantitatively   Extracts and filtrates of the culture of the effective organism may be tested by serial broth dilutions, turbidimetric measurements, agar plate dilutions, and cylinder plate methods (Waksman, 1945)   Animal tests may supplement these methods after a nontoxic extract or filtrate has been prepared

Serial dilution methods and animal tests have been useful in measuring the reaction of antibiotic extracts on virulent human type tubercle bacilli   Bush, Dickinson, Ward, and Avery (1945) report the use of the cylinder plate method with the rapidly growing nonpathogenic strain of tubercle bacillus known as "607," but slowness of growth and difficulties in preparing suspensions of virulent human type tubercle bacilli are probably responsible for the fact that the cross-streak and the cylinder plate method have not, to the knowledge of the authors, been reported using these organisms

This paper reports three agar plate methods which have been used to select actinomycetes with antibiotic properties and to test quantitatively filtrates and concentrates derived from these

METHODS AND RESULTS

Cross-streak method   Thirteen strains of actinomycetes[2] were selected for the tests   Seven media were chosen which would promote the growth of the actinomycetes and to each of these were added glycerol to the amount of 2 per cent and agar to the amount of 1 5 per cent   The glycerol may be omitted but the growth of tubercle bacilli is slower   The complete formulae for these media follow [3]

Medium 1

Corn steep liquor (Corn Prod Ref)	1 0%
K₂HPO₄	0 2%
NaCl	0 5%
Cerelose (Corn Prod Ref)	1 0%

Medium 2

Corn steep liquor	1 0%
K₂HPO₄	0 2%
NaCl	0 5%
Maltose, tech (Difco)	1 0%

Medium 3

Curbay B-G (U S Indus Chem)	0 5%
Casamino acids (Difco)	0 5%
NaCl	0 5%
Cerelose	1 0%

Medium 4

B-Y fermentation solubles (Comm Solv Corp)	0 5%
Casamino acids	0 5%
NaCl	0 5%
Cerelose	1 0%

Medium 5

Beef extract (Difco)	0 3%
Peptone (Difco)	0 5%
Maltose	1 0%

Medium 6

Corn steep liquor	1 0%
K₂HPO₄	0 2%
NaCl	0 5%
Maltose cp	1 0%

Medium 7

Beef extract	0 3%
Peptone (Difco)	0 5%
NaCl	0 5%
Glucose	1 0%

These media were adjusted to a pH of 7 0, tubed in 40-ml amounts, and stored in the icebox until needed   When melted and poured into plates this amount of medium helped to provide for loss by evaporation   The actinomycetes were streaked on the agar plates with a 4-mm loop from a spore suspension made by pouring saline over a sporulating slant and loosening the spores with a loop, or directly from a more stable preparation made by mixing the spores in a gelatin suspension and drying   These plates were incubated at 24 C for 5 days, or until a streak of growth about 1 cm in width had been established

A thick suspension of the H37Rv strain of *Mycobacterium tuberculosis* was obtained by grinding a 14- to 21-day-old pellicle growth from a flask of Proskauer

moculum was necessary to give uniform streak growth

Streaks of H37Rv were made with a 4-mm loop at right angles to the actinomycete streak, and the plates incubated at 37 C The growth of tubercle bacilli was at a maximum in 2 to 3 weeks and appeared as a wide rugose band 2 or 3 times the width of the original inoculating loop The degree of inhibition of the tubercle bacillus was measured in millimeters from the edge of the actinomycete streak Where several streaks of the same strain of tubercle bacillus were made, the readings were averaged Several plates were also streaked with both H37Rv and H37RvR, the latter a strain of H37Rv which had been made resistant *in vitro* to more than 1,000 micrograms of streptomycin per ml of medium (Williston and Youmans, 1947) Figure 1, nos 1, 2, and 3, show results of cross-streaking actinomycete plate cultures with H37Rv and H37RvR

At the time the results were observed, the hydrogen ion concentration of the agar adjacent to the streak was determined in order to eliminate inhibition due to acidity alone The hydrogen ion concentration was determined by cutting out strips of the agar and dissolving them in distilled water in the cup of a Coleman electrometer Table 1 shows the width of the zone of inhibition (in mm) of the H37Rv strain of tubercle bacillus by 14 strains of actinomycetes on seven different media chosen because they favored growth and antibiotic production by the actinomycetes Table 2 shows the results obtained on two media comparing the degree of inhibition of growth produced by the actinomycetes on the virulent H37Rv and the avirulent 607 strain Not only are the organisms inhibited to a different degree, but several actinomycetes inhibited the virulent H37Rv and not the avirulent 607 strain Obviously, if only the avirulent strain were used in these tests, effective antibiotics might be missed

Cylinder plate method This method was used in an attempt to make quantitative studies on filtrates and extracts of cultures which had already shown inhibitory properties The medium used was a modified Proskauer and Beck synthetic medium to which was added 1 5 per cent agar Forty ml of the nutrient agar were first put in the plate and allowed to harden, and then a 4-ml quantity of the agar that had been seeded with 7 5 mg (Hopkins tube) of tubercle bacilli per ml of agar was poured over the surface This inoculum of tubercle bacilli for the seeded layer was ground with mortar and pestle until very smooth so that the opacity of the growth layer was uniform after incubation Stainless steel cylinders were dropped gently through a plastic "guide" onto the plates Into these cylinders were delivered the diluted extracts or filtrates The plates were incubated 2 or 3 weeks and the diameters of the zones of inhibition were measured in mm Similar pour plates were also made using the streptomycin-resistant strain of H37RvR The cylinders were refilled when necessary, from time to time, with the extracts or filtrates to replace loss of potency due to exposure at incubator temperature

Cylinder plates were made using four cylinders to a plate Two of the cylinders on each plate contained 10 and 5 micrograms, respectively, of strep-

tomycin pei ml These consistently gave zones of inhibition of tubercle bacilli of approximately 25 and 15 mm, respectively, and served as controls Filtrates and concentrates of the antibiotics to be tested were placed in two different
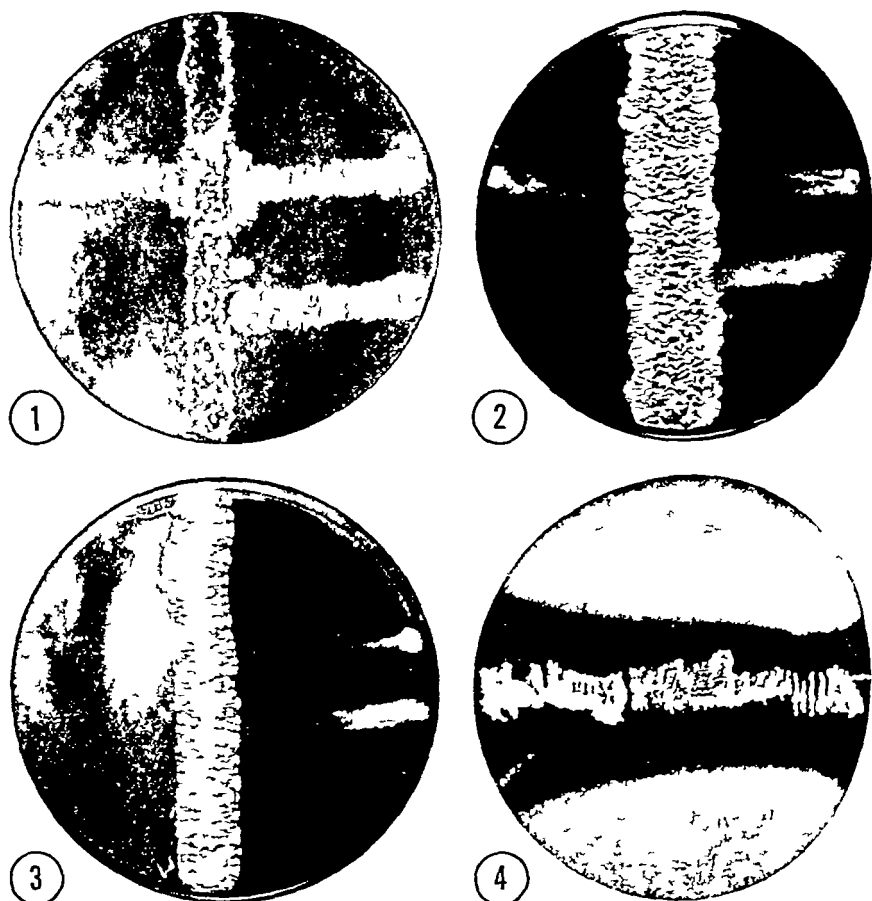
FIG 1 ACTINOMYCETE CROSS STREAKED WITH TUBERCLE BACILLUS
Vertical streak Actinomycete Upper horizontal streak H37Rv Lower horizontal streak H37RvR (resistant to >1,000 micrograms streptomycin)
No 1 No inhibition of either streptomycin-sensitive or streptomycin-resistant tubercle bacilli
No 2 Inhibition of streptomycin sensitive strain only
No 3 Inhibition of both streptomycin-sensitive and streptomycin-resistant strains
No 4 "Streak pour plate" seeded with H37Rv and cross streaked with an inhibitory organism

dilutions in the other cylinders A comparison with streptomycin could thus be established

Since the margins of the zones of inhibition were usually very fuzzy and indistinct, quantitative data were difficult to obtain In some cases, however, clear-cut zones were noted

Streak plates seeded with tubercle bacilli The plates seeded with tubercle

ACTINOMYCETE CULTURE NO	AMOUNT OF INHIBITION IN MILLIMETERS							
	Medium							
	1	2		3	4	5	6	7
	H37Rv	H37Rv	H37RvR*	H37Rv	H37Rv	H37Rv	H37Rv	H37Rv
1	>37	25	25	6	3	13	>32	
2	17	15	18	0	0	1	21	
3	20	0	0	6	7	1 5	17	
4	27	9 2	0	11	10	11	27	
5	14	11 3	6 3	11 3	0	7	15	
6	20	16	17 5	11	0	10	17	20
7	13	2 5	4	0	0	2	14	
8	0	0	0	0	0	23	0	0
9	17	19 6	0	8	10	15	15	
10	16	18	15	0	26	3	18	
11	0	0	0	0	0	0	0	0
12	20	3	4	10 6	11	8 3	12	`
13	15	15	20	12 5	11 6	18	14	15
S griseus†	20	12	0			12	20	0

* Resistant to streptomycin
† Furnished through the courtesy of Dr Selman A Waksman, New Brunswick, New Jersey

TABLE 2

Comparison of streak test results obtained with H37Rv and 607

ACTINOMYCETE CULTURE NO	AMOUNT OF INHIBITION IN MILLIMETERS			
	Medium 6		Medium 1	
	H37Rv	607	H37Rv	607
1	>32	7	>37	13
2	21 3	16	17 1	11 2
3	17 3	16	20 2	10 7
4	27	11 2	27	13 5
5	15	6	14	6 5
6	17	17	20	13
7	14	0	13	0
8	9	0	0	0
9	15	20	17	20 7
10	18 2	0	15 8	0
11	0	0	0	0
12	12	0	20	0
13	20 4	18 5	19 2	15
S griseus	20 2	21 5	20 5	?

bacilli prepared as described above were also used for streaking the actinomycete cultures   These were incubated first at 24 C for 5 days, then at 37 C for 2 weeks

If any of the actinomycetes possessed bacteriostatic properties, a zone of inhibi tion of the tubercle bacilli growing in the agar appeared next to the streak

Pour plates were seeded with both the resistant H37Rv and the sensitive strain Eight of the actinomycetes were cross-streaked and the inhibition zones measured   These inhibition zones were approximately the same as those obtained by cross-streaking the actinomycete with tubercle bacilli, as recorded in table 2 Figure 1, no 4, shows an inhibitory organism cross-streaked on a pour plate seeded with H37Rv

CONCLUSIONS

The streak plate method using the virulent type of tubercle bacillus (H37Rv) is useful for the testing of the antibiotic properties of actinomycetes   This gives a relatively rapid method for screening cultures in a search for new anti biotics   If a streptomycin-resistant strain of H37Rv is also streaked on the plates, cultures bearing a relationship to *Streptomyces griseus* may be detected

A smooth, opaque layer of growth may be obtained by seeding pour plates with H37Rv   Filtrates and concentrates in cups will give inhibition zones, though quantitative measurements are difficult to make because the zones are not always sharply defined

Pour plates, seeded with tubercle bacilli and streaked with actinomycetes, are useful in the search for cultures with tuberculostatic properties   The plates may be seeded with H37Rv or with H37RvR (resistant to streptomycin) and cross-streaked with various strains of actinomycetes

The avirulent, rapidly growing strain 607 is not suitable for this purpose, since some strains of actinomycetes which inhibit the virulent H37Rv strain do not inhibit, under the same conditions, strain 607

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A MORPHOLOGICAL VARIANT OF ESCHERICHIA COLI AND ITS RESISTANCE TO STREPTOMYCIN

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During a study of acquired resistance of bacteria to streptomycin a culture of *Escherichia coli* was found to give rise to a round-cell variant which could be maintained in culture. Although the effect of streptomycin on the original occurrence of these cells could not be definitely established, a study of the organism and the effect of streptomycin on it has been made. Round-cell forms of *Escherichia coli* have been reported by many workers as occurring in old cultures, in cultures under slightly toxic influences, and in cultures recently isolated from natural sources. In only a few instances have the round cells been found to develop, as such, unmixed with rods.

Coccoid cells very similar to those encountered here have been reported by Hussong (1933). He obtained a culture containing only round cells when a strain of *Escherichia coli*, which had been carried in lithium chloride broth, was transferred to lithium-chloride-free medium. The round cells returned to the rod form when grown again in a medium containing lithium chloride, but several transfers in lithium-chloride-free medium could be made before rods appeared in the culture. Mellon (1925a, 1925b) found round cells in a culture of *Escherichia coli* obtained from the urine of a patient who had been receiving urotropin and sodium acid phosphate. He was able to produce the same forms when a normal culture of *Escherichia coli* was grown in broth containing disodium glycerophosphate and sodium chloride. When this culture was transferred to a plain agar slant, only the round cells developed. On additional incubation these sprouted into coarse filaments and rods.

Dienes (1939, 1942, 1946) studied extensively the "large bodies" of many varieties of bacteria and their relation to the L type of colony. His studies on *Escherichia coli* were made with cultures from pathological urine specimens. He observed the germination of the large forms of *Escherichia coli* into both pleuropneumonialike and bacterial colonies. He states that in his experience the large bodies produced by toxic influences never germinate and reproduce. The naturally occurring round forms he studied were fragile, difficult to stain, and difficult to transfer. This is not the case with the cells reported here.

Alture-Werber *et al* (1945) reported bipolar rounded bodies which occurred in the urine of patients treated with penicillin. These reverted to typical *Escherichia coli* on culture. Price *et al* (1947) reported similar forms in cultures of *Eberthella typhosa* cultivated in broth containing streptomycin.

EXPERIMENTAL RESULTS

The culture of *Escherichia coli* used in this study had been carried in stock 11 years. The procedure for inducing resistance was to streak a series of meat

infusion agar plates containing streptomycin with an aqueous suspension of the organisms, incubate the plates 48 hours, select a colony from the plate containing the highest concentration of streptomycin, transfer it to a plain meat infusion agar slant, and after 24 hours' incubation repeat the process   It was on the sixth exposure to streptomycin that a colony, picked from a plate containing 100 micrograms of streptomycin per milliliter of agar, was found on transfer to plain agar to grow entirely as globular cells   Only the one colony was picked from this plate, later the culture from which this variant had come was restreaked and 8 colonies were picked and included in the resistance study   A total of 68 colonies were examined at various concentrations of streptomycin   None of these colony transfers showed a difference in morphology from the typical rod form of the parent culture   Another experiment was started with the parent culture in which 20 colonies were selected from each plate containing streptomycin and control colonies were selected from meat infusion agar   Four hundred and forty-three transfers from colonies growing on agar containing various concentrations of streptomycin and 449 transfers from colonies growing on plain agar were examined   All were rod forms   The variant form apparently was selected only by chance

The atypical culture when growing directly on agar containing streptomycin was a mixture of bizarre rods varying in size, some curved, branching, and Y forms, and a few round forms   A colony consisting of these cells when transferred to a plain agar slant grew only as round cells   These cells varied in size from about 1 to 7 microns and occurred singly, in pairs, chains, and groups, occasionally they gave the appearance of small cells budding from the larger ones   There was some variation in the density with which the different cells stained   All forms were gram-negative   The appearance of a large capsule surrounding the cells was characteristic of all stained preparations of the round cells   Figures 1 and 2 are photomicrographs of the two morphological forms [1]

The colonies of the variant on agar containing streptomycin were small, smooth, and nonmucoid, colonies of the round cells on plain agar were smaller than the parent *Escherichia coli* and were mucoid but not spreading   The mucoid character of the round-cell culture was most apparent on an agar slant

Studies were made of the cultural characteristics of the round-cell culture, of the parent culture, of a rod form of *Escherichia coli* which had become resistant to streptomycin, and of a culture of the variant after it had entirely reverted to the rod form   Reactions in nine sugars (glucose, maltose, lactose, sucrose, sorbitol, mannitol, salicin, raffinose, and xylose), tests for the production of nitrites, indole, and hydrogen sulfide, the liquefaction of gelatin, and the reaction in milk were the same for all cultures and were characteristic for *Escherichia coli*   The only differences noted were an occasional failure of the round form to grow in a few media and delayed growth of the variant in all media   After 24 hour incubation gas production by the round-cell cultures lagged considerably behind that by the rod cultures, at 48 hours it was equal

Antigenic studies were made on cultures of the parent strain, on the round

[1] The photomicrographs were made by Mr Norman Drake
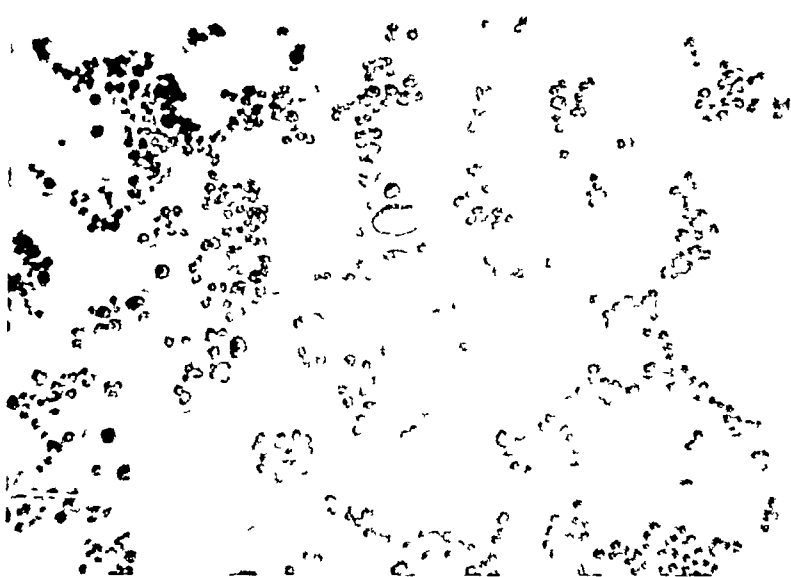
FIG 1 ROUND VARIANT OF ESCHERICHIA COLI 24 HOUR GROWTH ON PLAIN AGAR CA 1 000 ×
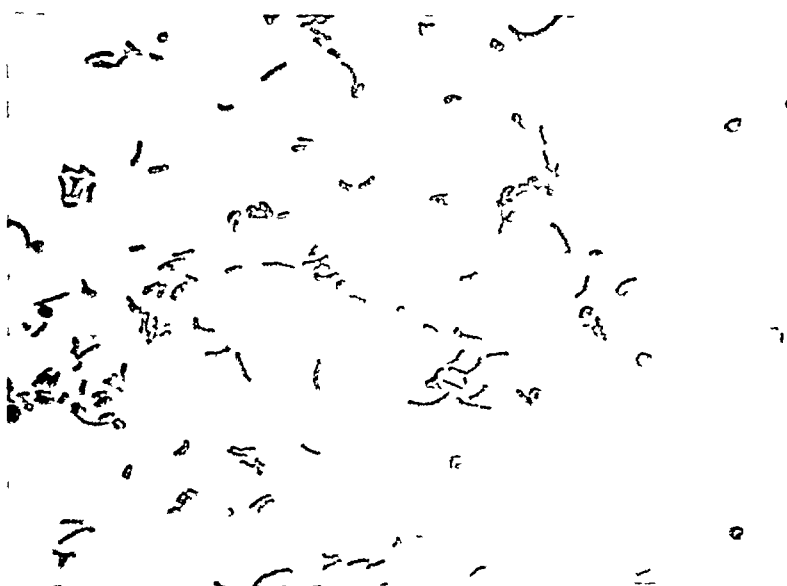
FIG 2 SAME VARIANT OF ESCHERICHIA COLI FROM A 48 HOUR COLONY ON AGAR CONTAINING STREPTOMYCIN CA 1,000 ×

form, which at that time was resistant to 10,000 μg streptomycin per ml, and on a rod form resistant to this same concentration of streptomycin   All antigens were agglutinated to titer with antisera against both the parent and resistant

rods   Antiserum against the round cells gave a titer of 1 1,600 with the homologous antigen but only 1 400 with the heterologous antigens   These cross reaction studies demonstrated an antigenic similarity of the two rod forms but an antigenic difference in the round and rod forms   Adsorption studies confirmed the similarity of the two rod forms and demonstrated the presence of an antigen in the round cells which was not present in the rods

The atypical round form was maintained by alternate transfers on agar containing streptomycin and plain agar   It has been carried through 67 exposures to streptomycin   For the first 22 exposures the plain agar slant transfer was found to contain only the globular cells   From the twenty-second to the sixty-seventh exposure plump rods were seen rarely in some of the agar slant cultures

A round-cell culture (20 exposures to streptomycin, resistant to 500 μg per ml) was transferred daily on plain agar and in plain broth   For the first 6 agar transfers and for 2 broth transfers the cultures were all round cells   After 14 transfers on agar or after 7 in broth the round forms had entirely disappeared and the cultures contained only small rods   For an additional 16 transfers no round cells were seen   Another round-cell culture much later in the series (of exposures to streptomycin, resistant to 10,000 μg per ml) required 23 transfers on plain agar before a preparation containing only rod forms was seen, and as this transfer series was continued it was found that a few round cells recurred

The fact that the variant form became resistant to streptomycin was thought to be of particular interest   However, the total number of exposures necessary before the round organism became resistant to 10,000 μg of streptomycin per ml agar was much greater than the number required for 11 other species of gram negative rods and the 8 colony subcultures of *Escherichia coli* obtained by the same method of random selection of one colony per plate   The parent strain of *Escherichia coli* was resistant to only 10 μg streptomycin per ml   At the sixth exposure, when the variant appeared, the rod had become resistant to 100 μg per ml   The variant continued to increase in resistance, on the eighth exposure it was resistant to 250 μg per ml and on the tenth to 500 μg per ml   Its resistance did not increase again until the thirty-fourth exposure, when it grew on 2,000 μg per ml, and on the next exposure, on 10,000 μg per ml   The round cell culture was then grown alternately on agar containing 10,000 μg streptomycin per ml and on plain agar for 38 more exposures, no higher concentrations were tested   With this continued culturing at the same high concentration the growth became much more abundant on the streptomycin agar, but the colonies transferred to plain agar grew poorly, sometimes failing to grow on the second transfer   Round forms still predominated on the agar slant transfers, but rod forms appeared more frequently   The abundantly growing organisms on agar containing streptomycin tended to be less bizarre, and very few round forms were present

Since attempts to obtain the variant form a second time from the parent culture under the stimulus of streptomycin had failed, it was thought that a similar variant might be selected from the reverted rod obtained by transfer of the round form on plain agar   As this series of transfers was made, each culture

was streaked on agar containing strept r g t   r   g
colonies were selected from these plates, transferred to slants, and examined
At first it appeared that the changing of reverted rod forms back to round forms
was being demonstrated since all colonies selected from the streptomycin plates
gave rise to round-cell cultures, whereas increasing numbers of the colonies
selected from plain agar were rods   But as the series continued with fewer and
fewer round forms being present in the transferred culture, it was observed that
fewer and fewer colonies appeared on the streptomycin plates at all, although
they all continued to be the atypical cells   It was found that the cultures
consisting of entirely reverted rods did not grow on streptomycin concentrations
above 100 μg per ml, whereas the round-cell cultures grew at 10,000 μg per ml or,
if selected from a lower concentration, grew at 10,000 μg per ml on the second
transfer to streptomycin   It could not be clearly demonstrated that reverted
rod forms were being changed to round forms, but it appeared that the round
forms remained resistant to high concentrations of streptomycin and that when
they became rod forms they lost that resistance

SUMMARY

A round-cell variant of *Escherichia coli* obtained from an agar plate containing
streptomycin has been described   This was apparently a chance selection as no
further similar strains were isolated   The variant and the parent strain gave
identical biochemical reactions   Antigenically the variant and parent strain
were not alike   The variant was maintained by alternate transfers on agar
containing streptomycin and on plain agar   Serial transfers on plain agar
produced a form which was identical to the parent strain culturally and mor-
phologically   No evidence was found that the round-cell form was a part of a
life cycle or a result of a sexual process

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ISOLATION OF TYPE B BOTULINUM TOXIN

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High yields of toxin have not been obtained with the nonproteolytic, and most of the proteolytic, strains of *Clostridium botulinum*, type B, in our culture collection   An exceptionally toxic type B culture designated as strain "okra" was received from the National Institute of Health   It is a proteolytic strain capable of producing, under certain conditions, one million minimum lethal doses of toxin for the mouse per ml of medium   From cultures of the "okra" strain a highly toxic and immunologically distinct protein has been isolated   Though this protein has not been crystallized it appears to be essentially a highly purified, single substance   The method of isolation for crystalline type A botulinum toxin of Lamanna, McElroy, and Eklund (1946) has not been applicable   This is probably a reflection of the physical and chemical differences that exist between the two serological types of toxin   Our methods and observations with the type B toxin are recorded in the following sections

Maintenance of the stock culture   The stock culture is kept in chopped-beef infusion medium consisting of meat fragments submerged in a double-strength beef infusion including 0 5 per cent sodium chloride and 1 per cent Difco proteose peptone   Once a month the culture is transferred using a 1-ml inoculum per test tube   Incubation is at 34 C for 48 hours, and storage is at room temperature

Medium and growth for toxin production   The organism is grown in 16-liter lots in 5-gallon pyrex glass carboys   The medium is composed of 1 per cent technical grade casein, 1 per cent alkaline-treated cornsteep liquor (47 to 52 per cent solids), and 0 5 per cent technical grade glucose   The cornsteep liquor is a filtrate of raw cornsteep treated by the addition of 1 part of water to 11 parts of cornsteep, alkalinizing with 40 per cent sodium hydroxide to pH 8 5, and heating at 65 C for a half hour   The casein is brought into solution by agitation at pH 9 5 or higher   The cornsteep liquor is added, and the pH adjusted to 7 2   The mixture is autoclaved at 120 C for 1 hour   Upon cooling, the proper amount of glucose solution, which has been autoclaved separately, is added

A flask with 500 ml of the same medium is preheated to remove dissolved air, cooled, and inoculated with the contents of a test tube of a 1-month-old stock culture   After overnight incubation at 34 C, 10 ml are transferred to 500 ml of medium in flasks corresponding in number to the carboys to be used for toxin production   These flasks in turn are incubated at 34 C overnight and then are used as inoculum, one per carboy   The carboys are incubated for 2 weeks at 34 C

As a consequence of vigorous fermentation during the first 24 hours of growth the pH drops rapidly to values of 5 3 to 5 5   The casein comes out of solution both as a sediment and as a thick, firm pellicle floating at the surface as a result

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of the entrapment of gas bubbles   At the beginning of the third day a decrease
in the evolution of gas and a rise in pH are noticeable   The pH continues to
rise slowly to 6 3 to 6 5 and may reach a value as high as 6 7 in two weeks' time
The decrease in acidity is accompanied by digestion of the casein   The bulk of
the casein becomes soft in consistency, friable, and smaller in quantity   By the
time the pH has risen to 6 0, toxin production in quantity becomes evident
A maximum titer (1 million MLD per ml or greater) is generally obtained
within 10 days of incubation   No exact time for maximum accumulation of
toxin can be stated as it has varied for each batch studied   As long as the pH
does not rise above 6 5, there seems to be little loss of toxicity   Some batches
have been incubated for 22 days without reduction in titer   The digestion of
casein proceeds beyond the period of maximum toxin production and is pre
sumably responsible for the continual decrease in acidity   It is of some advan
tage in the purification procedure to get rid of as much of the casein as possible
by means of the natural proteolysis

   Determination of toxicity   The toxicity of all preparations is determined by
intraperitoneal injection of 0 5-ml quantities of solution into 20-gram white
mice (± 2 g)   The mice are observed for a period of 4 days   The term MLD is
used in this report to signify the least amount of material killing all mice injected
(usually 4 mice per dilution) LD_{50} is calculated by the method of Reed and
Muench (1938) and is based on the use of 6 to 10 mice per dilution

   In making up dilutions of toxic solutions for titration purposes, 0 2 per cent
gelatin buffered at pH 6 5 by the use of 1 per cent phosphate salts has been
employed as diluent   It is a peculiarity of the toxin that it is relatively insoluble
at pH values above 4 5   Therefore, to keep the toxin in solution during dilution,
if a solution contains more than 5×10^6 MLD per ml, it is more satisfactory to
dilute with acidified water in the lower dilutions and with gelatin diluent in the
higher dilutions

   Flocculation tests with commercially available horse antitoxins were studied
briefly and discarded as an impractical means of following toxin concentration
The chief reason for this lies in the relative insolubility of the toxin at pH values
greater than 4 5   Inasmuch as flocculation tests are generally performed near
the neutrality point, the appearance of a flocculus with the toxin may be an index
of loss of solubility rather than of a specific serological reaction   It is highly
probable that the nature and quantity of other proteins present affect the
solubility of this toxin   Depending on the stage of purity and the character of
the serum in use, flocculation could, therefore, at times be the result of loss in
solubility and at other times the result of the formation of specific toxin antitoxin
aggregates   Combined with our meager knowledge of the toxin-antitoxin reac
tion for the particular system, this complex state of affairs made the flocculation
test a less practical means of following toxin concentration than titration in the
mouse

ISOLATION PROCEDURE

   The method developed for the isolation of the toxin from the culture medium
is fundamentally a series of acid precipitations   Salting out was tried at vario

toxin from solution   Slight additions of salt, such as 1 per cent saturation with $(NH_4)_2SO_4$ or NaCl to 0 1 M concentration, result in large decreases in solubility This is different from the experience with type A toxin of Lamanna, Eklund, and McElroy (1946)   The following is a description of the procedure   Unless noted otherwise, work was conducted at room temperature

Step 1   Acid precipitation of toxin from culture medium   The 14-day-old cultures are acidified with a strong acid (2 N HCl) to pH 4 0   As a result the bacterial cells, undigested casein, toxin, and other acid-precipitable material slowly settle out   The carboys are permitted to stand overnight for the purpose of collecting as much of the insoluble matter as possible   The supernatant is siphoned off, and the acid precipitates are pooled and freed of liquid by cen-trifugation (1,800 rpm in 250-ml cups)   The recovery of toxin in the acid mud may be 90 per cent or more effective   With some batches of culture pH 4 0 has not given quantitative recovery   It must be emphasized that the solubility of the toxin is probably greatly influenced by other proteins and products of bacterial growth   Inasmuch as the bacterial culture is a dynamic system, and all the variables affecting growth and proteolysis are not rigidly controlled, differences in composition between batches may exist at the time of acid pre-cipitation of the cultures   Be this as it may, the empirically established fact is that an acid pH value exists at which the solubility of the toxin in the culture medium is at a minimum   This value is pH 4 0, or a neighboring one   Snipe and Sommer (1928) and Sommer (1937) made the original observation that botulinus toxins are precipitated from medium by acid

Step 2   Extraction of the toxin from the acid mud   The precipitate from step 1 is resuspended in distilled water to one-fortieth the volume of the mother culture This reduced volume will be referred to as the original volume   A strong acid (2 N HCl) is added to bring the pH to 2 0   As much as possible of the undis-solved material is centrifuged off   The remainder is removed by filtration through a filter paper of fine porosity, which was previously wet with water acidified to pH 2   The clear filtrate contains about 80 per cent of the toxin

At this stage, before filtration but after centrifugation, if the suspension is rotated an anisotropic appearance is noted   Under the microscope the sus-pended material is observed to be a mixture of amorphous and extremely small needle-shaped material   It gives typical chemical tests for a protein   The specific gravity of this material must be nearly that of the suspending solvent, for it can be removed only with great difficulty by repeated high-speed centrifu-gation   A Sorval angle centrifuge rotating at 13,000 rpm or the multispeed attachment of the International Equipment Company's refrigerated centrifuge (model PR-1) has been used   The anisotropic material is insoluble at all pH values studied (2 0 to 7 0)   It remains finely divided at pH 2 0 to 4 5, aggregates above 4 5, and stays so up to pH 7 0, the highest pH value studied   This phenomenon is curious because the purified toxin itself in fairly concentrated solution remains soluble up to pH 4 5 and comes out of solution in clumps at higher pH values   The anisotropic material is not considered to be toxin since its potency per milligram of nitrogen is considerably lower than that for the

purified toxin, but it is definitely toxic   Repeated washing with acidified water
does not entirely remove its toxicity   Thus, if the material itself is not toxic
it strongly adsorbs toxin   The color of this material is nearly white in contrast
to the purified toxin which has a grayish-yellow cast   The material must be
associated with the growth of the organism, inasmuch as it is not obtainable
upon similar treatment of unautoclaved and autoclaved medium in which the
organism has not been grown

The choice of distilled water acidified to pH 2 for redissolving the toxin from
the acid precipitate of culture rests on a series of experiments with the toxic
acid-precipitated mud which showed   (a) The toxin was rapidly detoxified at

TABLE 1

Influence of pH, glycine, and salt on the resolution of toxin from an acid precipitate of medium

pH	SALT ADDED (FINAL CONC)	DILUTIONS (IN MILLIONS) OF SOLUTION KILLING 20 GRAM MICE			
		10	20	30	40
2 0	0	4/4 (26)*	2/4 (26) 2/4 (41)	4/4 (41)	2/4 (48) 1/4 (68)
	glycine 0 1 M	3/4 (26) 1/4 (41)	2/4 (26) 2/4 (41)	1/4 (41) 2/4 (48)	1/4 (48)
	NaCl 0 1 M	4/4 (26)	2/4 (26) 2/4 (41)	3/4 (41) 1/4 (48)	1/4 (48)
3 0	0	4/4 (26)	2/4 (26) 2/4 (41)	4/4 (41)	0/4
	glycine 0 1 M	3/4 (26) 1/4 (41)	3/4 (49) 1/4 (69)	0/4	0/4
	NaCl 0 1 M	0/4	0/4	0/4	0/4

* Ratio = $\dfrac{\text{no of mice dying}}{\text{no of mice injected}}$

Figure in parentheses is time in hours of observed death of mice

pH values above 6 5   Thus, a suspension of 20×10^6 MLD per ml lost none of
its potency in 3 days at room temperature at pH values of 2, 3, 4, and 5   At
pH 6 5 less than 10 per cent was lost, at pH 7 5 there was a loss of about 80 per
cent, and at pH 8 5 there was more than a 90 per cent loss   (b) Resolution was
poor in the pH range 4 5 to 6 5 and was quantitative only below pH 4   A
significant difference existed even at pH 3 and 2 (table 1)   (c) The addition
of sodium chloride or glycine to the extracting solvent decreased the amount of
toxin going back into solution, the effect being more notable at the higher pH
values (table 1)

Step 3 Precipitation of toxin from the extract of acid mud   The clear filtrate

igerator temperature   A flocculent precipitate, which is collected by centrifugation, forms

Recovery of toxin in this step has varied considerably   As the toxin is purified, it seems to become progressively more soluble at pH values on the acid side of the isoelectric range   This would definitely point to impurities as influencing the solubility of the toxin   If, because of variations between cultures, the nature and quantities of impurities vary, then the differences in recovery of toxin from separate batches at this stage are explicable   More recently it has been found that precipitation at pH 5 0 will result in higher and more consistent yields

Step 4   Washing of the precipitate of step 3   The precipitate of step 3 is washed by resuspension for 5 to 10 minutes in one-fourth original volume of a solution of 1 5 M NaCl at pH 2 0   The washed toxin is recovered by centrifugation   The solubility of the toxin in this solvent is low, being less than 100,000 MLD per ml

Step 5   Reprecipitation of the toxin   The centrifuged material of step 4 is redissolved in one-fourth original volume of acidified distilled water at pH 2 0   Difficulty with resolution of the toxin will be experienced if much sodium chloride is carried over from the preceding step   The toxin is then reprecipitated by bringing the solution to pH 5 0 to 5 5 and collected by centrifugation

The precipitate of step 5 represents purified toxin   It should be noted that except for the original toxic culture medium the solutions of the toxin are worked with at pH values on the acid side of the isoelectric zone   The method permits recovery of 50 per cent or more of the toxin

PROPERTIES OF PURIFIED TOXIN

The purified toxin is an odorless, slightly grayish-yellow-colored solid   Solutions appear yellow brown in color   During electrophoresis the color travels with the toxin boundary and shows no tendency to separate from it   No characteristic absorption spectrum is shown in the range from 800 to 340 mμ It reacts positively in qualitative tests for protein such as the biuret, ninhydrin, Millon's, xanthoproteic, and Hopkins-Cole   The Molisch test for carbohydrate is negative   The phloroglucinol test for nucleic acid is negative, and the orcinol test (Bial's reagent) slightly positive or negative   The absorption spectrum with ultraviolet light shows no evidence of the presence of nucleic acid   Absorption is maximum at 277 mμ Extinction plotted against wave length gives a curve typical for a simple protein (figure 1)   Chemical analyses for iron and metals precipitable by H_2S were negative   Thus no evidence for the presence of a prosthetic group has been obtained   The microkjeldahl nitrogen of toxin dried at 120 C in a vacuum oven is 15 5 per cent   Amide nitrogen has not been detected   Nitrogen of free amino groups was found to be 5 9 per cent of total nitrogen

An estimate of molecular size was made using the Northrop and Anson (1929) sintered glass membrane diffusion apparatus and their method of calculation The cell constant was determined by the use of NaCl   The diffusion constant

was calculated from analyses for nitrogen diffusing from the cell at 20 C in 12-hour intervals   The toxin was dissolved in acidified water at pH 2   The diffusion constant was 0 0624 cm^2 per day or 7 22 $\times$ 10^{-7} cm^2 sec^{-1} Assuming a spherical shape this indicates a radius of 2 9 $\times$ 10^{-7} cm and a molecular weight of about 60,000   This is in contrast to 900,000, the figure obtained by Putnam *et al* (1946), and 1,200,000, obtained by Kegeles (1946) by different method, for the molecular weight of crystalline type A toxin
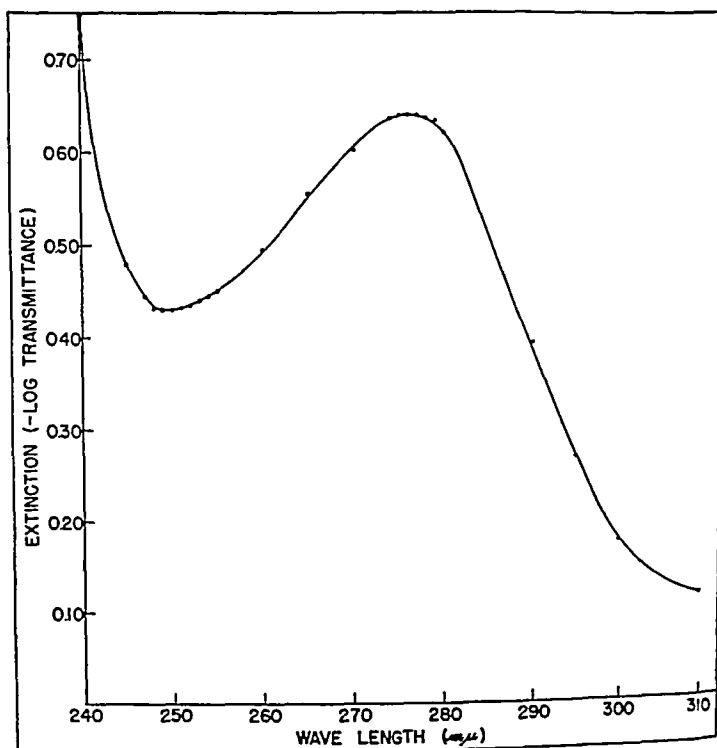
FIG 1 ULTRAVIOLET LIGHT ABSORPTION SPECTRUM OF PURIFIED TYPE B TOXIN

It is difficult to place the toxin in the American system of classification of the proteins   On heating, it is denatured and will coagulate above pH 4 5, but it does not have easily classified solubility properties   Electrophoretic mobility shows that pH 4 5 and lower values represent the acid side of the isoelectric range   The toxin is quite soluble in water on the acid side of its isoelectric range and only slightly soluble on the alkaline side   At pH 5 0 and 5 5 at 7 C, 0 043 mg of toxin nitrogen per ml was found in solution   At 20 C at pH 6 0, only 0 056 mg of nitrogen per ml was present   To date no electrophoretic studies between pH 4 5 and 7 0 have been possible because of inability to get sufficient material into solution

Unlike the globulinlike type A toxin, purified B toxin in water suspensions does not significantly increase in solubility upon the addition of salts   On the oth

with increasing additions of NaCl to the medium after toxin production has occurred results in an initial rise in solubility, followed by a decrease. At the same time, the pH of minimum solubility shifts to more acidic values. Thus without addition of salt the minimum solubility is at pH 4 0, but with 2 M NaCl pH 3 0 is the point of minimum solubility. The changes in solubility that are being measured in the case of table 2 represent toxicities of a few hundred thousand LD_{50} per ml, that is, extremely small concentration

TABLE 2

Influence of NaCl on solubility of toxin in mother culture at three pH values

SALT ADDED (FINAL CONC)	pH	TOXICITY OF DILUTIONS (IN THOUSANDS) OF SUPERNATANTS AFTER CENTRIFUGING OUT INSOLUBLE MATTER IN CULTURE				
		50	100	200	400	800
0	4 5	1/2*	3/4	2/4	0/4	0/4
	4 0	0/2	0/4	0/4	0/4	0/4
	3 0	1/2	4/4	4/4	2/4	0/4
0 1 M	4 5	2/2	4/4	3/4	4/4	0/4
	4 0	2/2	3/4	1/4	0/4	0/4
	3 0	2/2	3/4	4/4	4/4	0/4
0 5 M	4 5	1/2	4/4	4/4	3/4	0/4
	4 0	1/2	4/4	4/4	1/4	0/4
	3 0	2/2	4/4	4/4	2/4	4/4
1 0 M	4 5	2/2	4/4	3/4	4/4	2/4
	4 0	2 2	4/4	2/4	0/4	0/4
	3 0	2/2	4/4	4/4	4/4	0/4
2 0 M	4 5	2/2	4/4	4/4	3/4	0/4
	4 0	2/2	2/4	0/4	0/4	0/4
	3 0	0/2	0/4	0/4	0/4	0/4

* Ratio = $\dfrac{\text{no of mice dying}}{\text{no of mice injected}}$

differences. A milliliter of solution of 100,000 LD_{50} contains 5×10^{-4} mg of toxin nitrogen or a concentration of toxin of approximately 0 0003 per cent

Electrophoretic studies in glycine buffer at pH 1 8 reveal a single boundary on the ascending side. In addition to the major boundary a small boundary, which rapidly moves off the field of view, occurs on the descending side and does not reappear on reversal of the current (figure 2). Electrophoresis at pH 3 8 in glycine buffer showed a single moving boundary, the fast-moving boundary noted on the descending side at pH 1 8 was not seen. Whether the latter is an anomaly or has some special significance remains to be determined

Identity and potency of toxin. Toxin in culture medium and purified toxin were typed by using commercially available horse antitoxins in mouse protection
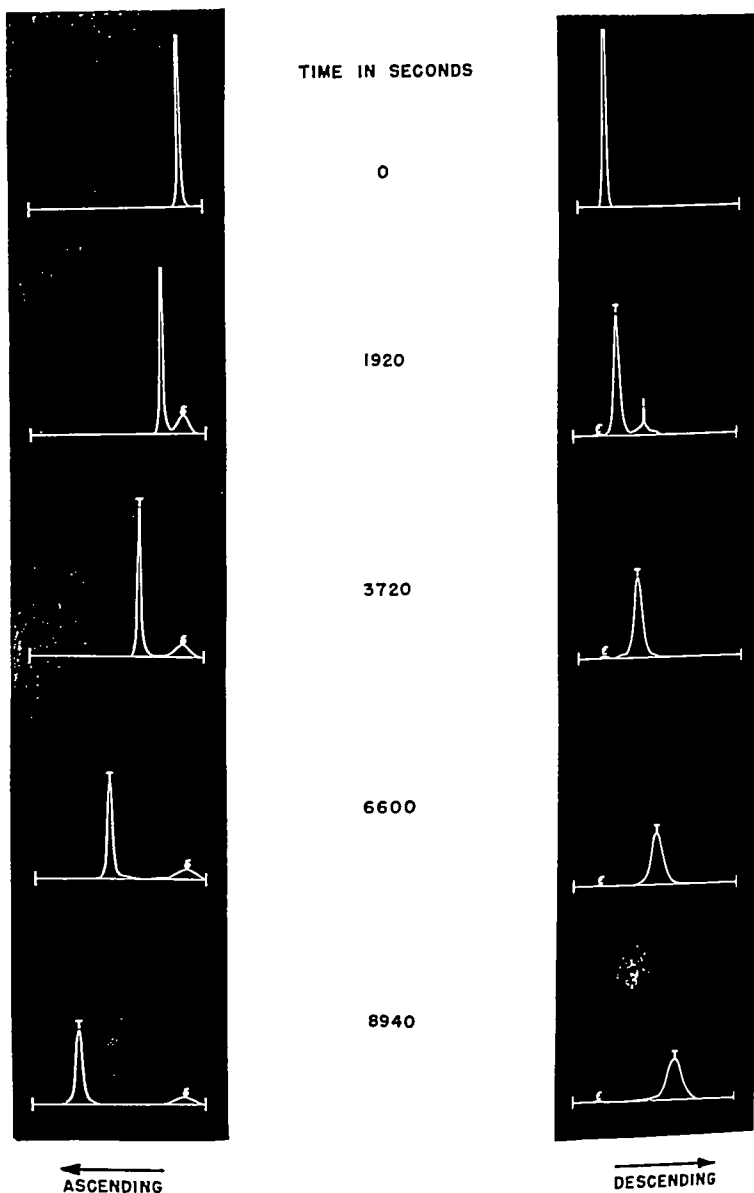
FIG 2 ELECTROPHORETIC PATTERN OF PURIFIED TYPE B BOTULINUM TOXIN (CONC 0 64%) DISSOLVED IN 0 1 M GLYCINE-HCl BUFFER AT pH 1 8 AT 3 9 C The moving boundary marked T is the toxin At 1,920 seconds the measurement mobility was 10 1 × 10⁻⁵ cm²/sec/volt on the descending side and 11 6 × 10⁻⁵ cm²/sec/vol on the ascending side Note a second moving boundary on the descending side labeled 1 in the 1,920 seconds' photo This boundary rapidly moved off the field of view so that 1 is no longer visible in the 3,720 seconds' photo Its mobility was 25 3 × 10⁻⁵ cm / cc/vol For this run the field strength was 4 31 volts per cm and the current 25 milliampere The stationary delta and epsilon boundaries are marked accordingly The illustration composite of drawings made from tracings of the original photographs

tests Monovalent types A and C antisera did not protect, but type B mon valent antisera did Rabbit antisera available as a result of immunization w

protective activity   Immunologically, there can be no doubt as to the identity of the toxin produced by the strain "okra"

On titration of one batch of the toxin in the mouse and the guinea pig, the following values were obtained

$$6\ 2 \times 10^{-9} \text{ mg nitrogen} = LD_{50} \text{ per 20-g mouse,}$$
$$31\ 2 \times 10^{-9} \text{ mg nitrogen} = LD_{50} \text{ per 300-g guinea pig,}$$
$$310 \times 10^{-9} \text{ mg nitrogen} = LD_{50} \text{ per kilo mouse,}$$
$$103 \times 10^{-9} \text{ mg nitrogen} = LD_{50} \text{ per kilo guinea pig}$$

These figures show that on a body weight basis the guinea pig is only three times as sensitive to the toxin as the mouse   This conclusion is in sharp contrast to the recent report of Stevenson, Helson, and Reed (1947) that the guinea pig is 6,000 to 8,000 times as sensitive as the mouse to the type B toxin   These workers have used strains other than "okra" and relatively impure toxin   The identity of similar serological types of toxin from different bacterial strains has been assumed, but final proof will rest with studies on pure materials isolated from different strains

Separately prepared batches of purified toxin have given values of toxicity from 5 to 9 $\times$ 10^{-9} mg of nitrogen per mouse LD_{50} This is of the same order of magnitude as $4\ 5 \times 10^{-9}$ mg nitrogen obtained with crystalline type A toxin   On a weight basis the two toxins appear equipotent, but if future research confirms the finding that the B toxin is 10 or more times smaller in molecular size, then on a molar basis the B toxin is considerably less toxic

The stability of the purified B toxin has presented itself as a serious problem   It appears more labile than the impure material   As a result we have not succeeded in storing solutions in the refrigerator for periods greater than two weeks without serious losses in potency   Interestingly enough, one batch of reduced potency studied appeared to have unaltered electrophoretic properties at pH 2

SUMMARY

A method for the purification of the type B toxin from the proteolytic "okra" strain of *Clostridium botulinum* is described   Essentially the purification depends upon working with the toxin on the acid side of its isoelectric zone and upon a series of acid precipitations   The purified toxin appears to be a slightly colored, simple protein, soluble in water on the acid side of the isoelectric range and relatively insoluble on the alkaline side and within the isoelectric range   Slight additions of salt do not favor increased solubility of the purified toxin

Serologically, chemically, and physically the purified B toxin differs from type A crystalline toxin   Its toxicity per milligram of nitrogen is only slightly less than that of the type A, but on a molar basis, it would appear to be 10 times less potent   By the intraperitoneal route the guinea pig is about three times more susceptible to the toxin than is the white mouse

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STUDIES ON POLYMYXIN AN AGAR DIFFUSION METHOD OF ASSAY

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Bacillus polymyxa was originally detected as a producer of an antibiotic (Stansly, Shepherd, and White, 1947) by the fact that surrounding the colony of the organism was a clear zone of agar, whereas the remainder of the agar plate was covered with a layer of growth of the test organism   Obviously the antibiotic diffused through the agar and prevented growth of the test organism up to a point where its concentration fell to a value below that required for this inhibition   It was, therefore, anticipated that it might be possible to develop an agar diffusion method to assay for potency similar to the well-known methods for penicillin (Abraham *et al*, 1941)

It was soon apparent, however, that the methods used for the assay of penicillin were not strictly applicable to the assay of polymyxin   For example, in a preliminary experiment using filter paper disks saturated with solutions of polymyxin, insignificant zones of inhibition were obtained using *Escherichia coli* as the test organism, even with high concentrations of the antibiotic   Incubation of the plates was at 37 C   Experiments were then undertaken, many of them qualitative in nature, to determine the conditions necessary for obtaining large, sharply defined inhibition zones   When these conditions were determined, it was anticipated that it might then be possible to relate zone size to antibiotic concentration

The following variables were studied (1) the type of medium in base and seed layers, (2) the concentration and amount of agar in the base and seed layers, (3) the pH of the medium in the seed layer, (4) the incubation time and temperature, (5) the effect of surface-active agents, (6) the pH of antibiotic solutions, (7) the type of test organism, (8) the inoculum size in the seed layer, and (9) the use of filter paper disks as opposed to ceramic cylinders   In all of these studies with the exception of that involving test organisms, *E coli* (MacLeod) was the assay organism used

Brief statements of the results obtained will be followed by an account of the final method which evolved and the type of data which was obtained   A statistical analysis of the assay and a method for estimating the error of any assay will be given

The type of medium in base and seed layers   A variety of media and combinations of nutrients at different concentrations were tried   Few were equal to and none superior to "TSP"[1] (at a concentration recommended by the manufacturer)

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[1] Trypticase soy-phosphate medium (Baltimore Biological Laboratory)

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from the standpoint of zone size and definition   Since, in addition, "TSP" is a ready-made dehydrated material, it was selected as the assay medium of choice

Concentration of agar and amount of agar in base and seed layers   To favor diffusion in the seed layer it was thought desirable to reduce the agar concentra tion to a minimum   At first 1 5 per cent agar was used and later 1 2 per cent Four ml of agar was considered to be the smallest amount that could be con veniently spread over the base layer   The base layer was more or less arbitrarily set at 20 ml of 2 per cent agar   Some variation of this was tried but resulted in no particular advantage

pH of medium in seed layer   A comparison of zones obtained with the seed layer adjusted to an initial pH of 5, 7, and 9 was made   Growth but no zones were obtained at pH 5   The zones at pH 9 were smaller than those at pH 7 The pH of the seed layer medium was therefore set at 7

Incubation time and temperature   The importance of the proper incubation conditions for the success of the assay cannot be overemphasized   It is worth repeating that incubation at 37 C, no matter what other conditions were imposed, led only to insignificant zones of inhibition even with high antibiotic concentra tions   It was surmised that these insignificant zones at 37 C were due to one of two factors or, perhaps more correctly, to an interaction of the two   These factors were, first, and possibly most important, the extremely rapid growth rate of *E coli* (Mason, 1935) and, second, the relatively slow diffusion of polymyxin

To combat the first, recourse was had to lower incubation temperatures   This had the desired effect of increasing markedly the zone of inhibition   However, no single incubation temperature between 15 C and 30 C was entirely satis factory because the depressing effect on growth resulted in poorly defined and uneven zones   On the other hand, an initial period of low temperature incuba tion followed by a period of higher temperature incubation was found to be a satisfactory compromise   It appeared that the low temperature incubation slowed down bacterial growth to such an extent that the relative rates of growth of *E coli* and of diffusion of the antibiotic were in favor of the latter   After the antibiotic had been given an opportunity to diffuse before appreciable growth started, it was possible to continue incubation of the plates at a higher tempera ture without adversely affecting the zone of inhibition   The 37 C incubation may be looked upon as merely a device for smoothing and accentuating the contrast at the edge of the zone by providing favorable conditions for the rapid multiplication of the bacteria surrounding it   As finally evolved, the low tem perature incubation was at 25 C for 18 hours   This was followed by 6 hours at 37 C, thus permitting an assay to be completed in 24 hours

Effect of surface-active agents   The second factor influencing zone size was considered to be the diffusion of the antibiotic   It was thought that substances which reduce interfacial tension might increase diffusion, thus increasing the zone size and hence the sensitivity of the assay   The following experiments illustrate the results obtained   All measurements were an average of three replicate zones

Experiment 1   "Aerosol OT"[2] and "tween 60"[3] were incorporated in the seed

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[2] American Cyanamid Company
[3] Atlas Powder Company

Table 1 shows that "tween 60" had the effect of ıncreasıng the sıze of the zone
On the other hand, "aerosol OT" faıled to do thıs   Of ıncıdental ınterest ıs
the apparent antagonıstıc effect of "aerosol OT" on the antıbıotıc   In the con-
centratıons used, neıther "aerosol OT" nor "tween 60" had any observable
effect on the growth of *E colı*

TABLE 1

Effect of "aerosol OT" and "tween 60" on the zone of ınhıbıtıon ın the assay of polymyxın

CONC REAGENT	ZONE DIAMETER IN MM	
	Aerosol OT	Tween 60
per cent		
0 5	0 0	26 75
0 05	0 0	25 5
0 005	18 0	23 0
0 0005	18 0	22 25
0 00005	22 0	
0 0	22 0	

TABLE 2

Effect of "tween 60" and "tween 80" on zone of ınhıbıtıon ın the assay of polymyxın

CONC. REAGENT	ZONE DIAMETER IN MM	
	Tween 60	Tween 80
per cent		
4 0	30 0	30 0
2 0	30 0	29 0
1 0	30 0	29 25
0 5	29 25	29 0
0 25	29 25	29 0
0 125	30 0	29 0
0 05	28 5	29 0
0 005	27 5	28 0
0 0005	27 25	27 25
0 00005	27 0	27 5
0 0	26 5	

Experıment 2 ıs a comparıson of "tween 60" and "tween 80"[4] under ıdentıcal
condıtıons   The results are summarızed ın table 2, whıch confirms the general
effect noted ın experıment 1   "Tween 60" and "tween 80" were approxımately
equıvalent ın actıvıty

Experıment 3 ıs a tıtratıon of polymyxın comparıng the zones obtaıned wıth
"tween 80" (1 per cent) and those obtaıned ın the absence of any surface-actıve
agent   Table 3 ındıcates the results   These confirm the results of experıments 1
and 2 and, ın addıtıon, demonstrate the ıncreased sensıtıvıty obtaıned wıth the

[4] Atlas Powder Company

surface-active agent That is, under these conditions 8 units per ml could be detected in the presence of "tween 80," whereas only 16 could be determined in its absence

TABLE 3

Titration of polymyxin with and without "tween 80"

CONC POLYMYXIN	ZONE DIAMETER IN MM	
	Control	Tween 80
*units/ml**		
128	26 7	28 8
64	24 3	25 7
32	19 7	23 25
16	16 7	20 0
8	0	16 0
4	0	0

* For a definition of the unit see "Procedure "
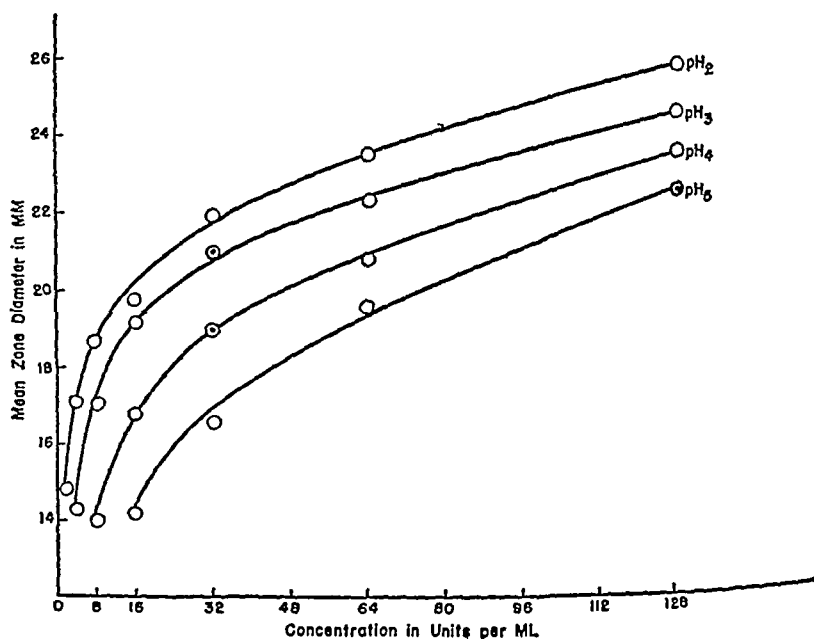
FIG 1 EFFECT OF pH OF POLYMYXIN SOLUTIONS ON ZONE DIAMETERS

Effect of pH of antibiotic solutions Up to this point, solutions of polymyxin for the purpose of potency determinations were made up in 0 02 M phosphate buffer, pH 7 0 It was now desired to determine the effect, if any, of varying the pH of such solutions upon the quality and diameter of the inhibition zone.

Experiment 1 Solutions of the antibiotic were prepared in 0 85 per cent saline adjusted to pH 3, 5, 7, and 11 and diluted from 512 units per ml to 2 units per ml in saline of the respective pH The zone diameters were approximately equivalent at corresponding concentrations for the solutions of pH 5, 7, and 11, the

ι   ι   ι ˽ι, ν   ι˽ιˈ˽ ˽ ι̥ιι ˽ ρˍι ι   were required for   o+ ⁻ preparations

Experiment 2   Solutions of polymyxin were made up in and diluted with glycine-HCl buffers[5] at pH 2, 3, 4, and 5, and assayed   The results are best illustrated graphically and are shown in figure 1   In this experiment it was possible to determine 2 units per ml of polymyxin in solution at pH 2, whereas only 16 units per ml could be determined at pH 5   The buffers alone had no apparent effect on the growth of the test organism

Solutions of polymyxin are quite stable except in alkaline regions (Stansly *et al*, 1947)   Therefore it seems unlikely that the effect of the pH of the antibiotic solution on zone size is a reflection of pH stability of the antibiotic   These effects are likewise unrelated to any interaction with the filter paper of the disks since the same effects were demonstrated with ceramic cylinders   The underlying reason for the apparent increased diffusion or activity of polymyxin in the agar medium with decreasing pH is at present obscure

In addition to increasing the sensitivity of the assay, another advantage was apparent with antibiotic solutions at the lower pH's in the glycine-hydrochloric-acid buffers   The zones appeared to be more consistently round and regular than those obtained with the same material at pH 7 in phosphate buffer or saline

The test organism   A desirable test organism for the assay is one which is very sensitive to the antibiotic, gives good growth in 24 hours, and is preferably non-pathogenic   *E coli* (MacLeod) satisfied all these requirements   However, it seemed worth while to look for an organism with such a growth rate that the entire assay could be carried out at a single temperature   Several were investigated (e g , *Salmonella pullorum*), but none proved satisfactory for this purpose

Effect of inoculum size   The smaller the number of bacteria in the seed layer, the larger the resulting zone of inhibition, and hence the greater the sensitivity of the assay   If carried far enough, however, a point is reached at which increased sensitivity can be achieved only at the expense of definition and regularity of the zones   The optimum inoculum was obtained by diluting a 24-hour, 37 C broth culture of *E coli* (MacLeod) to a final concentration of 1 2,000 in the agar seed layer

Filter paper disks vs ceramic cylinders   No differences were found between disks and cylinders other than that ascribable to the differences in their respective diameters   Thus, a zone obtained with a given concentration of polymyxin measured 25 mm with the disk and 21 mm with the cylinder, but the difference in the diameters of the disk and cylinder was exactly 4 mm   From the standpoint of simplicity and convenience, the disk seemed far superior to the cylinder method and was selected as the procedure of choice

PROCEDURE

The standard   Lot 5 (crude, dry acetone precipitate) was set aside as the standard preparation of polymyxin for assay purposes and stored in a desiccator (under calcium chloride) in the refrigerator

[5] Sorensen's buffer mixtures (Gortner's *Outlines of Biochemistry*, 2d ed , p 123)   This buffer at pH 2 was also used for the routine assay as described under "Procedure "

The unit   Repeated assay of the standard by the agar streak method gave an inhibition end point of 8 micrograms per ml with *E coli* (MacLeod) as the test organism   Therefore, the unit of activity was considered as equivalent to the activity of 8 micrograms per ml of the standard preparation

Preparation of the standard solution   The standard (204 8 mg) is dissolved in 100 ml of 0 05 M glycine-hydrochloric-acid buffer, pH 2, giving 256 units per ml Tests have shown that such a solution is stable indefinitely in the refrigerator Further dilutions of the standard are made in the same buffer

Preparation of samples for assay   Solid samples are dissolved in 0 05 M glycine hydrochloric-acid buffer, pH 2, and dilutions are made with the same buffer Aqueous liquids (e g , fermentation liquors) are first diluted with an equal quantity of 0 1 M glycine-hydrochloric-acid buffer, pH 2, and further dilutions are made with 0 05 M buffer   Nonaqueous solutions of polymyxin are preferably evaporated to dryness and then treated as solid samples   Insufficient experience with nonaqueous solvents or mixed solvents does not justify any statement of the validity of their use in the standard assay procedure

Preparation of plates   Twenty ml of 2 per cent TSP agar (pH approximately 7 3) are poured into petri plates and allowed to solidify   Four ml of 1 2 per cent agar containing 1 per cent "tween 80" and a 1 2,000 dilution of a 24-hour, 37 C TSP broth culture of *E coli* (MacLeod) are then spread over the base layer   The seed agar is dispensed with a 10-ml pipette from a single flask maintained at 48 C in a water bath   The plates are next dried in special trays, with lids raised, for 45 minutes in a dry 37 C incubator

Preparation of saturated disks   Three filter paper disks (Schleicher and Schuell no 740E, ½″ diameter) in a sterile petri dish are saturated with a single dilution (of either standard or unknown) by distributing 0 4 ml from a 1 ml pipette   Any excess is removed by touching the disk twice on a dry area of the plate   The saturated disks are then placed on the seeded agar by means of forceps   The distribution of the saturated replicate disks is dependent upon the manner of estimating potency, as will be brought out later

Incubation of completed plates   The completed plates are replaced in the special trays mentioned above and incubated overnight (16 to 18 hours) at 25 C with lids raised   The following morning the trays and plates are transferred to a well-humidified 37 C incubator and incubated for 6 more hours   The plates are then taken out and the zone diameters measured in mm in any convenient way

DETERMINATION OF POTENCY

Method 1   In this method three disks are saturated with a dilution of the standard solution and placed upon a seeded plate   This provides one point of the standard curve   Other points are obtained from similar plates containing additional dilutions of the standard   Only one dilution of the unknown is used Three disks are saturated with it and also placed upon a seeded plate   Upon each plate, in addition, is placed a single disk saturated with one particular concentration of the standard, e g , 128 units per ml, the purpose being to enter

The triplicate disks of each standard dilution are averaged, and a standard curve relating potency in units per ml (plotted logarithmically) to diameter of zone of inhibition is constructed   Such a standard curve is shown in figure 2 Fermentation liquors also give a linear relationship
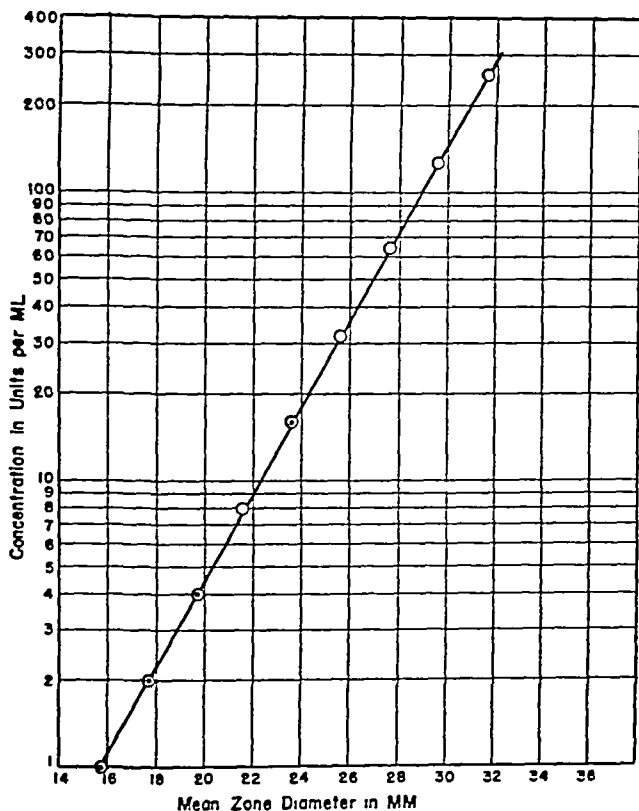
FIG 2 STANDARD POLYMYXIN ASSAY CURVE

This method has been successfully used when a relatively small number of assays were to be made   Certain difficulties became apparent when a large number of assays were made because of the lapse of several hours between the time the first and last samples were completed   For example, suppose that the plates for the standard curve are prepared first and that the last sample is assayed after 1 hour   During this time growth could take place at an appreciable rate (depending on the room temperature) in all the plates, but diffusion of the antibiotic only in those plates already completed (including the standard plates)   Therefore, the resulting standard curve could not be used for estimating the potency of the last sample without incurring a considerable error, since equal concentrations of antibiotic would give different zone sizes under these conditions

The following experiment illustrates this type of behavior Upon each of
three seeded plates was placed a filter paper disk saturated with 64 units per ml
of a standard solution   After standing at room temperature for an hour, each
of the three plates received another freshly saturated disk of 64 units per ml of
the standard solution   After the usual incubation the zone diameters were
measured, with results given in table 4   This table shows that the average
difference between the two treatments was 3 0 mm   If it were imagined that
the standard applied after 1 hour was in reality an unknown sample, its potency
would have been reported as about 33 per cent too low

Method 2   To obviate the foregoing difficulty the method of the Food and
Drug Administration for the assay of penicillin (Knudson and Randall, 1945)
seemed admirably suitable   In this method each assay is independently per
formed in conjunction with its own standard   No standard curve in the usual
sense is used   The results are calculated by the use of formulae or nomographs
derived from consideration of the straight-line log dose vs response relation

TABLE 4

Effect of delay in applying saturated disk to seeded plate

	ZONE DIAMETER (MM) UPON APPLYING DISK	
	Immediately	After 1 hour
	26	23
	27	24
	27	24
Avg	26 6	23 6

ship, such as exists with penicillin and polymyxin   In the procedure now in use,
two disks of the standard, each saturated with a different concentration, and
two disks of the unknown, also of different concentrations, are placed upon a
single seeded plate   It is essential that the ratio of potencies of the two standard
disks be the same as the two unknown disks   In our case the ratio is usually 4
Three replicate plates are used, rather than four as recommended for penicillin
by the Food and Drug Administration

A word should be said about the assay of fermentation liquors as opposed to
that of concentrates   Concentrates invariably give zones of excellent definition
This is not always the case with fermentation liquors   Occasionally, and this
seems to be particularly true of "stationary" fermentation liquors, diffuse or
double zones, or both, are obtained which render measurement difficult and un
certain   The concentration process evidently removes the interfering sub
stance   Fortunately aerated cultures are relatively free of this defect   When
it does occasionally exist, it is usually of no serious consequence

STATISTICAL ANALYSIS OF THE MICROBIOLOGICAL ASSAY OF POLYMYXIN

Since it was anticipated that the error in the assay of fermentation liquors
would be greater than that in other samples (e g , concentrates), the data from

, , , y of preparations   Ten assays in each category were taken at random from over a period of a month and subjected to an analysis of variance, which took the form shown in table 5

It is apparent that seven degrees of freedom per assay, derived from the interactions, were available for the estimate of error   The standard deviation, based on the total interactions of the ten assays (70 degrees of freedom) in each category, was found to be 0 434 mm for beers and 0 304 mm for all other samples The χ^2 test revealed a significant difference between the two values

<div align="center">TABLE 5</div>

<div align="center">Form of analysis of variance for the assay of polymyxin</div>

SOURCE OF VARIATION		DEGREES OF FREEDOM
Type	Source	
Main effects	Standard vs unknown (preparations)	1
	Low conc vs high conc (concentrations)	1
	Replicate plates	2
First order interactions	Preparations $\times$ concentrations	1
	Preparations $\times$ plates	2
	Concentrations $\times$ plates	2
Second order interaction	Prep's $\times$ conc's $\times$ plates	2
Total		11

The standard error of a log ratio of potency, S_M, $= S \, h \, i \, t \, \sqrt{\dfrac{B^2 + D^2}{B^2}}$ (Bliss and Marks, 1939), wherein

S = 0 434 or 0 304 mm
h = 1 (for a two-dose test)
i = 0 602 (log of the interval of doses, i e , log 4)
t = 1 994 for 19 in 20 odds
$D^2 = \dfrac{V^2}{12}$, where $V = \Sigma(V_H + V_L) - (S_H + S_L)$ for the triplicate plates

The symbols V_H, V_L, S_H, and S_L are the zone diameters of high and low unknown and high and low standard, respectively (Knudson and Randall, 1945)

$B^2 = \dfrac{W^2}{12}$, where $W = \Sigma(V_H + S_H) - (V_L - S_L)$ for the triplicate plates of an assay (Knudson and Randall, 1945)

Substituting these values, $S_M = \dfrac{K\sqrt{V^2 + W^2}}{W^2}$, wherein

K^1 = 1 81 (beers)
K^2 = 1 27 (all other samples)

$100 \left[\text{antilog} \left(\dfrac{K\sqrt{V^2 + W^2}}{W^2} \right) - 1 \right]$ expresses the error of the assay as a per-

TABLE 6

*Error of assay as a percentage of potency**

(95 per cent probability for beers)

±	W→ 20	21	22	23	24	25	26	27	28	29	v
0	23	22	21	20	19	18	18	17	16	15	15
1	23	22	21	20	19	18	18	17	16	15	15
2	23	22	21	20	19	18	18	17	16	15	15
3	23	22	21	20	19	18	18	17	16	15	15
4	24	23	22	20	20	18	18	17	17	16	15
5	24	23	22	20	20	18	18	17	17	16	15
6	24	23	22	21	20	18	18	17	17	16	15
7	25	23	22	21	20	18	18	18	17	16	15
8	25	24	23	21	20	19	19	18	17	16	16
9	25	24	23	22	21	19	19	18	18	16	16
10	26	25	23	22	21	19	19	18	18	16	16
11	27	25	24	22	21	20	19	18	18	16	16
12	27	26	24	23	22	20	20	18	18	16	16
13	28	26	25	23	22	20	20	19	18	17	16
14	29	27	25	24	23	21	20	19	19	17	16
15	29	28	26	24	23	21	21	19	19	18	17
16	30	29	27	25	24	21	21	20	19	18	17
17	31	29	27	25	24	22	21	20	20	18	18
18	32	30	28	26	25	22	22	20	20	18	18
19	33	31	29	27	25	23	22	21	20	18	18
20	34	32	29	27	26	23	23	21	21	19	18
21	35	33	30	28	26	24	23	22	21	19	18
22	36	33	31	29	27	24	24	22	21	20	19
23	37	35	32	29	28	25	24	22	22	20	19
24	38	36	33	30	29	25	25	23	22	20	19
25	39	37	34	31	29	26	25	23	23	21	20
26	41	37	35	32	30	27	26	24	23	21	20
27	42	39	35	32	31	27	26	24	24	21	21
28	43	40	37	33	31	28	27	25	24	22	21
29	44	41	37	34	32	29	28	25	25	22	21
30	45	42	38	35	33	29	28	26	25	23	2?
31	46	43	39	36	33	30	29	26	26	23	22
32	48	44	40	37	34	30	29	27	26	24	23
33	49	45	41	37	35	31	30	28	27	24	23
34	50	46	42	38	36	32	31	28	27	24	23
35	52	48	43	39	37	32	31	29	28	25	24
36	53	49	44	40	37	33	32	29	28	25	24
37	55	50	46	41	38	34	33	30	29	26	25
38	56	51	47	42	39	35	33	31	29	26	25
39	57	52	48	43	40	35	34	31	30	27	25
40	59	54	49	44	41	36	35	32	31	28	25

$$* \ 100 \left[\text{Antilog} \left(\frac{1 \cdot 81 \ \sqrt{V^2 + W^2}}{W^2} \right) - 1 \right]$$

V	W→20	21	22	23	24	25	26	27	28	29	30
±											
0	16	15	14	13	13	13	12	12	11	11	10
1	16	15	14	13	13	13	12	12	11	11	10
2	16	15	14	13	13	13	12	12	11	11	10
3	16	15	14	13	13	13	12	12	11	11	10
4	16	15	14	14	13	13	12	12	11	11	10
5	16	15	15	14	14	13	12	12	11	11	10
6	16	15	15	14	14	13	13	12	11	11	11
7	17	16	15	14	14	13	13	12	11	11	11
8	17	16	15	14	14	13	13	12	12	11	11
9	18	16	15	14	14	13	13	13	12	11	11
10	18	16	16	15	14	13	13	13	12	11	11
11	18	17	16	15	15	13	13	13	12	12	11
12	19	17	16	15	15	14	13	13	13	12	11
13	19	18	16	16	15	14	14	13	13	12	11
14	20	18	17	16	16	14	14	13	13	12	11
15	20	18	18	16	16	15	14	13	13	12	11
16	21	19	18	16	16	15	14	14	13	13	12
17	21	19	18	17	16	15	15	14	13	13	12
18	22	20	19	17	17	15	15	14	14	13	12
19	22	20	19	18	17	16	15	15	14	13	13
20	23	21	19	18	18	16	15	15	14	13	13
21	24	21	20	18	18	16	16	15	14	14	13
22	24	22	20	19	18	17	16	15	15	14	13
23	25	23	21	19	19	17	16	16	15	14	13
24	26	23	22	20	19	18	17	16	15	14	13
25	26	24	22	20	20	18	17	16	16	15	14
26	27	24	23	21	20	18	18	16	16	15	14
27	28	25	23	21	21	19	18	17	16	15	14
28	29	26	24	22	21	19	18	17	16	15	14
29	29	26	24	22	22	19	18	18	16	16	15
30	30	27	25	23	22	20	19	18	17	16	15
31	31	28	26	23	22	20	19	18	17	16	15
32	32	29	26	24	23	21	20	19	18	16	16
33	33	29	27	24	24	21	20	19	18	17	16
34	33	30	27	25	24	22	20	19	18	17	16
35	35	31	28	26	25	22	21	20	19	18	16
36	35	31	29	26	25	23	21	20	19	18	16
37	37	32	29	27	26	23	22	21	19	18	17
38	37	33	30	27	26	23	22	21	20	18	17
39	38	33	31	28	27	24	23	21	20	19	18
40	39	34	31	29	27	24	23	22	20	19	18

$$* \; 100 \left[\text{Antilog} \left(\frac{(1\,27\,\sqrt{V^2 + W^2})}{W^2} \right) - 1 \right]$$

centage of potency   Tables were prepared, one for beers (table 6) and one ie·
all other samples (table 7), in which the error (for odds of 19 in 20) as a pe:
centage of potency was calculated for values of V from 0 to 40 and W from 20 to
30, covering perhaps 99 per cent of all combinations of V and W likely to occu.
In practice W does not vary appreciably from 24, whereas V varies in accordance
with the relative potency of unknown and standard   It may be pointed out
that the factors V and W are easily obtained for each assay and are prerequisites
for calculating the potency of an unknown, in addition to the error

The determination of potency and the estimation of its error are based on
assumed parallelism of the log concentration vs the response curves of standard
and unknown   In isolated instances this assumption may not be justified,
and hence the estimation of potency and its error would be invalid   Therefore,
a test for departure from parallelism, i e , a significant interaction of preparation
$\times$ concentration, is made routinely for each assay   The variance of this inte.
action divided by the triple interaction variance gives the required factor fo
the test   If this ratio is 4 35 or greater, then there is only a 5 per cent chance
that the standard and unknown slopes are really the same, and it is presumed
that they are significantly different   The factor 4 35 is obtained from a table of
"F" for 1 and 20 degrees of freedom

For routine purposes the test for significance of slope difference may be simpli
fied   Thus, it was calculated, on the basis of the considerations above, that if
$(S_H - S_L) - (V_H - V_L)$ is 4 0 or more for beers and 2 0 or more for all oth
samples, then a significant departure of parallelism between standard and un
known slopes is presumed to exist, and the particular assay is discarded   A
small percentage of the assays fall in this class

Experience has shown that the usual error for a 95 per cent probability is
$\pm$20 per cent for beers and $\pm$15 per cent for all other samples   This error can
be further reduced by repeating the assay   The average percentage of err.
thus obtained divided by the square root of the number of repetitions give. the
percentage of error of the average potency

From time to time it may be desirable to check the standard deviations upon
which the error is based   Quality control methods (Knudson and Randall,
1945), when justified, would also be of value

SUMMARY

The factors influencing *Escherichia coli* inhibition zones produced by the ant·
otic polymyxin are considered   An agar diffusion method of assay is descri :
and a statistical analysis presented   As customarily used, the error for a 95 p
cent probability is in the neighborhood of $\pm$15 to 20 per cent   This c. br
further reduced, if desired, by appropriate replication

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THE RELATION BETWEEN OXYGEN CONSUMPTION AND THE UTILIZATION OF AMMONIA FOR GROWTH IN SERRATIA MARCESCENS

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The metabolic differences between assimilating and nonassimilating cells have been the subject of several recent investigations   In this connection a stimulation of glycolysis when ammonia is added to yeast has been shown by several authors (Runnstrom, Brandt, and Marcuse, 1941, Winzler, Burk, and du Vigneaud, 1944)   Armstrong and Fisher (1947) *have demonstrated a comparable increase in the rate of oxygen consumption by* Escherichia coli *when the assimilation of ammonia is taking place*   It follows from these observations that it may be possible to determine the amount of glycolysis or carbon dioxide produced and the amount of oxygen consumed during the assimilation of known quantities of the nitrogen source

It will be shown in the present work that in the bacterium *Serratia marcescens* the rate of oxygen consumption is also higher during the assimilation of ammonia than it is in the absence of such assimilation   Following the uptake of the last of the ammonia, as noted for *E coli* by Armstrong and Fisher, the rate at which oxygen is consumed by the bacterium falls sharply to a lower rate, which is typical of resting cells   This lower rate is a definite percentage of the higher one regardless of how much growth has taken place   It is, therefore, permissible to calculate the resting rate which corresponds to each rate observed for the growing cells   Any oxygen consumed in excess of the amount expected for resting cells must then be associated with the assimilation of ammonia   This quantity of oxygen has been measured along with the quantity of ammonia actually assimilated

MATERIALS AND METHODS

The preparation and maintenance of the bacteria   The organism used in this investigation was the bacterium *Serratia marcescens* (*Bacillus prodigiosus*), American Type Culture Collection no 990   It was maintained on a synthetic medium, modified from that used by Bunting (1940), having the following composition   glycerol 1 25 g, citric acid 4 g, K_2HPO_4 9 g, $MgSO_4$ $7H_2O$ 0 5 g, and NH_4Cl 1 g, adjusted to pH 7 with NaOH, and made up to 1 liter with distilled water   Twenty-five g of agar were added and the medium was autoclaved at 15 pounds' pressure for 15 minutes

The bacterial suspensions for the respiration experiments were prepared as follows   A slant was inoculated from 1 loopful of bacteria, it was incubated for

17 hours at 30 C (a preliminary experiment showed that this temperature gave better growth than 20 or 37 C), and the growth was then washed off into 0 07 M potassium phosphate buffer at pH 7   The suspension was made up to the desired concentration, about 1 × 10⁹ bacteria per milliliter, by the reflectometer (Libby, 1941)

The measurement of oxygen consumption   The rate of oxygen consumption was measured in a Warburg respirometer (Umbreit, Burris, and Stauffer, 1945) at 30 C, with air being used as the gas phase and with the vessels shaking through an arc of 5 cm approximately 100 times per minute   Under these conditions there was no indication that the concentration of carbon dioxide was a limiting factor. The vessels were prepared with 1 0 ml of the bacterial suspension plus 0 5 ml of solution A (i e , MgSO₄ 7H₂O 2 0 g, glycerol 20 g, sodium citrate 12 6 g, adjusted to pH 7 with HCl, and made up to 1 liter with distilled water) in the main space of the vessel, 0 5 ml of distilled water or a solution of ammonium chloride in the onset, and 0 3 ml of 10 per cent potassium hydroxide in the inset with filter paper

The determination of ammonia   For this analysis the bacteria were separated from the suspending medium by filtration through fritted glass filters (pyrex, no 36060, 15 UF) under reduced pressure   The filtrate was collected in 1 ml of 50 per cent (by volume) sulfuric acid

The ammonia in the filtrate was determined by a procedure essentially the same as that described by Peters and Van Slyke (1932) in connection with the determination of urea in urine   To the acid filtrate was added distilled water to a volume of 10 ml and then 5 ml of 5 N KOH   Air, after passage through 5 per cent H₂SO₄ , was drawn through the alkaline mixture and thence through 15 ml of 0 02 N HCl, the ammonia being trapped in the latter   The total ammonia thus collected was estimated colorimetrically using a Cenco Sheard Sanford photelometer, following the procedure outlined by Snell and Snell (1936), and using Jackson's modification of Nessler's reagent   This procedure can be used provided the quantity of ammonia present is not over about 12 × 10⁻² mg   It is reproducible to within about 0 25 × 10⁻² mg of ammonia in the sample filtered

Determination of total (Kjeldahl) nitrogen   The contents of the respirometer vessel were washed into 1 ml of the digestion mixture (1 part saturated K₂SO₄ 1 part concentrated H₂SO₄ , and a small amount of selenium powder, cf Snell and Snell, 1936) in a pyrex test tube   Two glass beads were added and a glass bulb was placed on top   The tube was heated vigorously over a microburner until the water had been boiled off and the contents of the tube had begun to fume, the flame was reduced, and the mixture was allowed to boil gently until it was well charred   When charring had taken place, the tube was cooled for about 30 seconds, and a few drops of 30 per cent H₂O₂ were dropped on the charred material   The mixture usually decolorized at once   It was then reheated, decolorized again if necessary, and finally boiled until it had remained clear for several minutes   This was taken as the end point of the digestion   The nitrogen then present as ammonium sulfate was determined exactly as described above for ammonium chloride

In order to establish the actual relationship between the uptake of ammonia and the rate of oxygen consumption, both processes were studied simultaneously The experiments were conducted as follows The respirometer vessels were prepared with the bacterial suspension and solution A in the main part of the vessel and with an amount of ammonium chloride (0 19 mg) which would sustain growth for only a few hours in the onset   After being shaken 1 hour in the constant temperature bath with the ammonium chloride in the onset, the bacteria
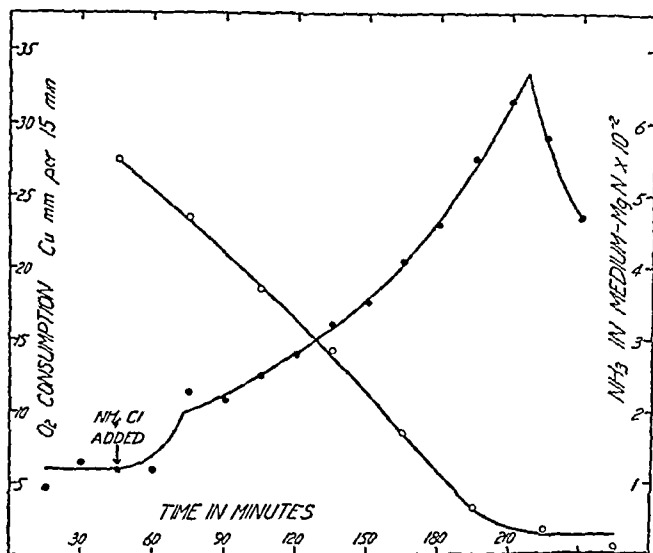
FIG 1 A typical experiment showing as a function of time
   (1) the rate at which oxygen disappears from a respirometer vessel containing cells of *S marcescens* (dots) and
   (2) the quantity of ammonia present in the medium (circles)
   Each point is the average result in two identical vessels   The ammonium chloride was added to the organisms from the onsets of the vessels at the point indicated

reached a "resting" state, and the rate of oxygen consumption was comparatively steady although decreasing very gradually with time [1]   The ammonium chloride was then added to the bacteria   Measurements of the rate of oxygen consumption were continued, and at intervals the contents of the vessels were analyzed for ammonia, one of the vessels being removed for this purpose immediately following the addition of the ammonium chloride, and others every few minutes thereafter

   The results of a typical experiment are shown in figure 1 in which the solid circles indicate the rate at which oxygen was taken up in the respirometer vessels, whereas the open circles indicate the ammonia remaining in the medium   Before

   [1] Any nutritive materials washed off the culture slants with the organisms were apparently in such low concentrations as to be completely metabolized during this initial hour in the respirometer

the addition of ammonia, the rate of oxygen consumption is relatively constant and there is, of course, no growth   Upon adding ammonia, however, the medium becomes one which will support growth—it is, in fact, the one on which the organism was being maintained   At this point the rate of oxygen consumption rises quite abruptly and after some 20 to 30 minutes reaches a value which is nearly double the initial value   There then ensues a period during which the logarithm of the rate of oxygen consumption is a linear function of time   The curve drawn through the observed points in figure 1 during this phase of the experiment was obtained by calculation presuming that the logarithm of the rate is a linear function of time   It is clearly a good representation of the data   It undoubtedly represents the gradual increase in the quantity of bacterial protoplasm in the respirometer vessel, as others have noted (Greig and Hoogerheide, 1941, Hershey and Bronfenbrenner, 1938)   From it the time for the bacterial mass to double, that is, for the logarithm of the rate of oxygen consumption to increase by the logarithm of 2, may be determined   This averaged 72 minutes (standard deviation,[2] 6 minutes) in 10 experiments

It will be noted in the figure that the amount of ammonia present in the suspending medium decreases steadily throughout the experiment   It does so, of course, because it is taken up by the cells for elaboration into new protoplasm The curve describing the utilization of the ammonia actually, therefore, represents the time course of the formation of new protoplasm   It is in fact a "growth curve "

As the concentration of ammonia approaches zero, the rate of oxygen consumption quite suddenly falls, just as has been described for $E\ coli$ (Armstrong and Fisher, 1947), to a relatively steady value   The latter is illustrated in the experiments of longer duration which are shown in figure 4 and which are to be discussed in detail below   In 9 experiments this resting rate was on the average 56 6 per cent of the maximum rate seen in the respirometer (standard deviation, 3 6 per cent)   This steady (strictly, slowly declining) rate represents the resting rate which is characteristic of the amount of bacterial protoplasm now present in the respirometer vessel   Since the ammonia has been exhausted, it is evident that no appreciable uptake of ammonia can occur after the rate of oxygen consumption starts to decrease   It follows, then, that these organisms consume oxygen at either of two different rates, just as $E\ coli$ does, depending upon whether or not assimilation of ammonia is occurring

This conclusion arises again when the rate of oxygen consumption and the rate of ammonia utilization are compared   As noted above, there is a rapid rise in the rate of oxygen consumption when the ammonia is first added   The rate of ammonia utilization, however, does not show any evidence of a similar initial spurt   It seems again, therefore, that in order to grow under these conditions the cells present must consume oxygen at a rate above that characteristic of a resting phase

2 The standard deviation was taken as $\dfrac{(x - \bar{x})^{\cdot}}{N - 1}$ where x is the result of one experiment, $\bar{x}$ is the mean of the results, and N is the number of experiments

to estimate the difference between the activity and resting rates at the beginning of nitrogen assimilation   This results, firstly, from the lack of information about the existence of an initial lag period in the growth curve and, secondly, from the fact that the chemical systems involved here have considerable inertia, as indicated by the observation that, following the exhaustion of the nitrogen source, the rate of oxygen consumption does not decrease instantaneously
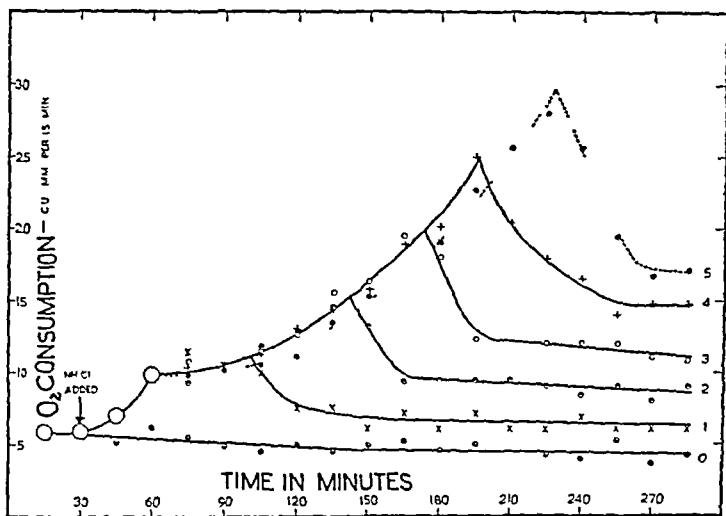
FIG 2   The time course in a typical experiment of the rate at which oxygen disappears from a respirometer vessel containing cells of *S marcescens*   Each point is the average result in two identical vessels   As in figure 1 ammonium chloride was added to the organisms from the onset of the vessel at the point indicated   The numbers appearing at the right-hand end of each curve give, in hundredths of milligrams, the actual quantities of nitrogen added as ammonium chloride to the several vessels

During the first hour the rates of oxygen consumption in the various vessels are essentially identical   Observations during this interval have, therefore, been indicated in the figure by single circles which have been made large enough to encompass all of the observations made at each time   In this particular experiment, although it was not usually so, the data for the highest concentration of ammonium chloride differed slightly from the curve describing the remainder of the points [3]   To avoid confusion, therefore, the trend of these points is indicated by dashes

To provide further information about the changes in the rate of oxygen consumption when ammonia is added or exhausted, the consequences of adding different quantities of ammonia were studied   These experiments were made by placing aliquots of bacterial suspension in each of several respirometer vessels, in the onsets of which different amounts of ammonium chloride were placed   As in the experiment described in figure 1, the resting rate of oxygen consumption was determined, and then the ammonia was tipped into all the vessels   Typical observations of the rate of oxygen consumption in an experiment of this kind are given in figure 2

[3] This would result if, by accident, fewer bacteria had been placed initially in one of these vessels

In every case, when the ammonium chloride is added there is an initial rap d
increase in the rate of oxygen consumption   This is followed by the gradual
logarithmic increase already described   After the ammonia is exhausted, th⁻
rate of oxygen consumption falls to the lower resting rate
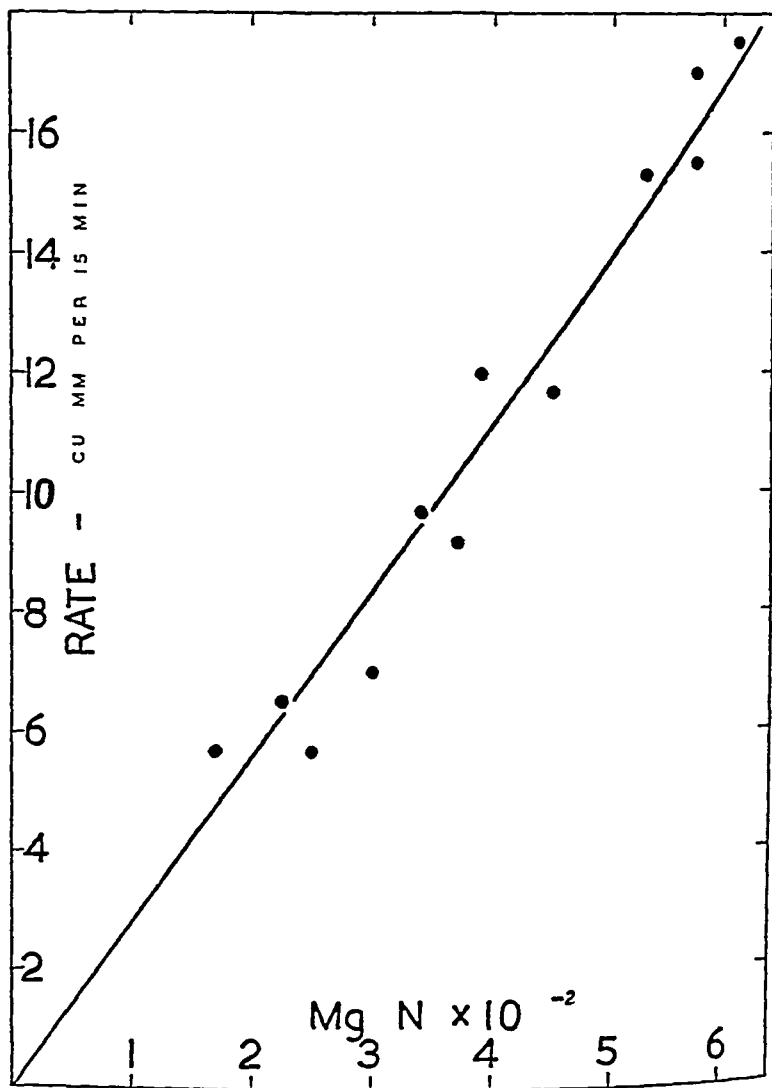
FIG 3 A typical experiment in which the rate at which oxygen disappears from a
respirometer vessel containing resting cells is shown as a function of the quantity of
bacterial nitrogen present in the vessel   Each point is the result for one vessel

It will be noted that the only significant differences between the several curve⁻⁺
in figure 2 are the durations of the logarithmic phase and the absolute level at
which the rates fall after exhaustion of the ammonia   As might be expected   ⁺
logarithmic phase lasts longer, and the final resting level attained is higher t⁻

a constant percentage of the maximum rates reached, the percentage being independent, therefore, of the amount of growth which had taken place   As noted above, the resting levels averaged approximately 56 per cent of the peak rate

At the termination of the experiments illustrated in figure 2, the contents of the vessels were analyzed for nitrogen   Determinations of the quantity of ammonia and total nitrogen in the suspending medium alone indicated that at this time all of the nitrogen present was in the cells   The analysis on the entire contents of the respirometer vessels thus measures the bacterial nitrogen present at this time   The latter can also, of course, be obtained by adding to the nitrogen present in the original aliquot of bacterial suspension, with which the experiment was begun, the amount of ammonia tipped into the vessel to initiate growth   In any case it is possible to compare the resting rates observed, after assimilation has ceased, with the amount of nitrogen present in the bacteria   This has been done in figure 3, and it is apparent there that the rate at which oxygen disappears in a respirometer vessel, containing resting cells, is directly proportional to the quantity of bacterial nitrogen which is present   It is to be noted that this is true even for the initial aliquots of bacteria, i e , before any growth occurs in the respirometers   Moreover, the line in figure 3 passes through the origin, indicating that the nitrogen content is an absolute measure of the rate of oxygen consumption (cf Hershey, 1939, Burris and Wilson, 1940)   It is quite definite, therefore, that the several different resting rates recorded in figure 2 indicate the presence of different quantities of bacterial protoplasm   It may be calculated from the data in figure 3 that, on the average, these bacteria consumed oxygen at the rate of $1\ 12 \times 10^3$ cu mm per hour per mg of nitrogen when suspended in solution A

It is now evident that for any particular rate of oxygen uptake along the logarithmic part of the curve in figure 1, there is a corresponding lower resting rate to which the rate at which oxygen is disappearing would fall if the ammonia were suddenly removed   This lower rate was shown above to be determined solely by the amount of bacterial protoplasm present   Since it forms a constant percentage (approximately 56 per cent) of the activity rate, it is possible to plot on a graph, such as that in figure 1, a line which shows the time course of the resting rate following the addition of ammonia to the cells   During the period of logarithmic growth the resting rate is 56 per cent of the activity rate   To obtain the resting rate during the initial rapid rise in the rate of oxygen consumption, the curve describing the time course of the resting rate during logarithmic growth may be extrapolated backwards   Similarly the resting rate during the fall in the rate of oxygen consumption, following exhaustion of the ammonia, may be obtained by extrapolating backwards the nearly horizontal straight line which at the termination of the experiment describes the resting rate

A calculated line giving the time course of the resting rate has been plotted along with a set of experimentally determined rates in figure 4   It is evident that the area enclosed by the lines describing, respectively, the observed rate of oxygen consumption and that indicating the time course of the resting rate rep-

resents the volume of oxygen consumed by the growing cells in excess of that required by resting cells   It is an accompaniment of the growth process   More specifically, it is the amount of oxygen consumed during the assimilation of a known quantity of nitrogen in the form of ammonia   The number of oxygen atoms consumed during the assimilation of each nitrogen atom given in the form of ammonia may, therefore, be calculated   The average value found in 10 experiments was 2 19, the standard deviation of the individual values about this mean being 0 14   This value was observed to be independent of the quantity of ammonia assimilated for quantities varying from 0 012 to 0 06 mg
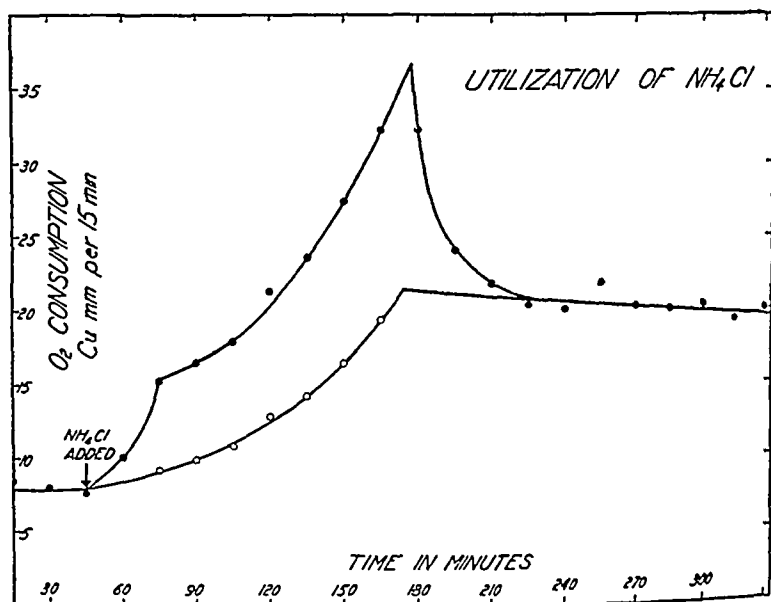
FIG 4   The time course in a typical experiment of the rate at which oxygen disappears from a respirometer vessel containing cells of S marcescens (dots)   Each point is the average of three identical vessels   Ammonium chloride was added at the point indicated   The calculated time course of the resting rate is indicated by circles

SUMMARY

The oxygen consumption of the bacterium Serratia marcescens was studied in both growing and resting cells, and the rate of oxygen consumption per milligram of bacterial nitrogen was found to be higher when the assimilation of ammonia was taking place

The extra oxygen used during the assimilation of the ammonia was determined It was found that 2 2 oxygen atoms were taken up for each nitrogen atom assimilated

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FURTHER STUDIES ON THE IMMUNIZATION OF RABBITS TO TOXIGENIC CORYNEBACTERIUM DIPHTHERIAE BY INJECTIONS OF NONTOXIGENIC DIPHTHERIA BACILLI[1]

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Frobisher and Parsons (1943) reported that rabbits injected with broth cultures of living avirulent (nontoxigenic) *Corynebacterium diphtheriae* developed significant resistance to subsequent injections of living cultures of virulent (toxigenic) *C diphtheriae*

Their experiments involved 21 immunized and 11 control animals These were tested with a challenge dose that was fatal to all of the 11 control rabbits, which died on an average of 3 6 days after the dose was administered [2] Of the immunized animals 4 survived, the other 17 dying after an average of 7 days In all, 48 per cent manifested some degree of resistance, including the 4 survivors Resistance was ascribed to mobilization of cutaneous defenses, which effected, not a neutralization of toxin by antitoxin, but toxin localization Antitoxin was not present Apparently resistance was related to a greatly enhanced tissue reactivity and was presumably engendered by somatic antigens of the bacilli against a heterologous antigen—the exotoxin

The present investigation was undertaken to extend and verify these observations and to collect further information regarding the phenomena observed

MATERIALS AND METHODS

Infusion broth and agar   These were prepared with veal or pork, according to the methods outlined in the *Manual of Methods* of the Society of American Bacteriologists, with the following modifications (1) Neopeptone (Difco) was used in 1 per cent concentration, and (2) the meat infusion was heated to 80 C before pressing out the juice

Synthetic medium   This was used in experiments to study the role of thiamine in the effectiveness of the antigens   The formula is given in the description of the experiments in which it was used

Cultures   The avirulent strains of corynebacteria (cd107b and My654a) used as antigens were the same as those used by Frobisher and Parsons (1943), and tests for avirulence and atoxigenicity were not repeated   The virulent strain (EHD70) used for challenge doses was also the strain used by these workers Broth cultures, 48 hours old, were used for both immunizing and challenge doses

Inoculations   Several immunization programs were conducted with variations

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[2] In later experiments using numerous controls no control animal has survived the same challenge dose

evidence of increased resistance as evidenced by the 5 4-day average survival time of the 14 remaining rabbits   All of the 10 control rabbits died within 5 days or less

A probable confirmation of the importance of *fresh* pork in the preparation of these antigens was later obtained inadvertently   Because of severe wartime shortages of meat fresh pork became unavailable   A preparation called, commercially, "pork-sausage," and probably consisting largely of corn meal and other nonporcine material, was used in cultivating antigens for one immunization experiment involving 12 rabbits   The results (table 3) were like those obtained with veal-grown antigens   At most only slight resistance was produced in the test animals   The average survival time was only 3 4 days as compared with 2 days for the controls   If the sausage contained fresh pork, which seems very unlikely, it must have been present in very small amounts, and its properties must have been modified by the spices and other materials mixed with it and by the processing to which it had been subjected

TABLE 3

Reaction of rabbits to a virulent challenge dose following immunization with avirulent antigen prepared with "pork-sausage" infusion broth

PERIOD OF IMMUNIZATION	RABBITS	SURVIVAL TIME	SURVIVAL TIME AVERAGE
weeks		*days*	*days*
5	12	2–6	3 4
Control	1	2	2*

* Compare also controls in tables 1, 2, 4, and 5

II Reactions to the Challenge Dose

The local reaction to the challenge dose in most of the animals immunized with organisms grown in a fresh pork base (not "pork-sausage") medium was characteristic   An area of very marked edema, 6 to 12 cm, often more, in diameter, developed within 24 to 48 hours   Sometimes the whole flank of the animal was involved   This was gradually absorbed over a period of several days, and a corresponding but somewhat smaller area of necrosis developed   In contrast to these edematous reactions, the control animals, as well as most of the animal injected with organisms grown in media other than fresh pork infusion (including "pork-sausage"), developed much smaller lesions with little or no edema and much less extensive necrosis   Apparently resistance was closely related to the extent of the skin reaction

As originally described, the resistant animals showed little or no evidence of general intoxication at any time, whether or not they survived, until a few hours before death if they died after several days   The controls and nonresistant (veal and "pork-sausage") animals were obviously ill within 24 to 36 hours after administration of the challenge dose   Evidently toxin was absorbed rapidly from the local lesion in the control and nonresistant animals but was held in slowly in the resistant animals

In order to have some confirmation of the observation that the survival of animals in these experiments is not dependent on the development in them of antitoxin, some of the test animals in this series were bled before receiving the challenge dose   The serum of 6 of the 8 animals surviving the challenge dose was examined and in each instance was found to contain less than 0 01 unit per ml   The sera were not assayed at lower levels

IV   Effect of Thiamine

From the results described above it was inferred that fresh pork contains some factor which is of critical significance in the antigenicity of avirulent diphtheria bacilli in regard to virulent diphtheria bacilli   Data on the amino acid and vitamin content of veal and pork were obtained from the American Meat Institute   According to these data an important difference between pork and veal is in the thiamine content, which is decidedly greater in pork

Further experiments (exp 6, 7, 8, 9, and 10) were conducted to verify the earlier results with veal-grown antigens and to determine whether or not thiamine had any influence on the phenomenon under study   The thiamine effect was studied with thiamine-enriched[4] veal infusion medium and with a synthetic medium developed in this laboratory and based on the method of Pappenheimer et al[5]   Efforts were made to use the media of Uschinsky (1893) and of Hadley

[4] One mg per cent thiamine chloride added before sterilization

[5] Sodium lactate	6 ml	Valine	1 g
Glucose	1 g	Leucine	500 mg
MgSO₄	1 g	Methionine	200 mg
K₂HPO₄	4 g	Tyrosine	100 mg
NaCl	6 g	Pimelic acid	10 mg
Tryptophane	200 mg	*Beta*-alanine	10 mg
Cysteine hydrochloride	200 mg	CuSO₄	10 mg
Glycine	200 mg	H₂O (dist)	1,000 ml
Glutamic acid	2 g		

Heat to dissolve
Adjust with n/1 NaOH to pH 7 8 or 8 0
Boil vigorously for 5 min
Add distilled water to restore volume
Cool to room temperature
Filter through a good grade of filter paper
Dispense and sterilize in the autoclave (15 lb, 20 min)
To each 100 ml of this base add aseptically 0 2 ml of vitamin solution 1 or 2

Vitamin Solution 1		*Vitamin Solution 2*	
Ascorbic acid	100 mg	To Vitamin Solution 1 add	
Niacinamide	40 mg	Thiamine chloride	350 mg
Riboflavin	8 ml	Sterilize by Seitz filtration	
(100 mg % in H₂O)			
Pyridoxine	1 ml		
(100 mg % in H₂O)			
Calcium pantothenate	2 ml		
(100 mg % in H₂O)			
H₂O	89 ml		

Sterilize by Seitz filtration

An effort to learn the nature of this factor served merely to demonstrate that thiamine is not the responsible agent

These studies have amply corroborated the earlier finding (Frobisher and Parsons, 1943) that under proper experimental conditions rabbits that receive repeated doses of cultures of avirulent diphtheria bacilli develop a resistance to, and in many cases survive, doses of virulent diphtheria bacilli that are invariably fatal to normal rabbits   Two important additional facts have also been established   (1) as between the media used here, a fresh pork base medium is essential to antigenic effect, (2) thiamine is not per se responsible for the antigenic effect

The implications of these findings are fairly obvious with respect to media used in the preparation of antigens heretofore regarded as of little efficacy, such as dysentery and cholera vaccine, etc , and the improvement of bacterial antigens already in use, such as typhoid and pertussis   The antigens might be made more effective by the inclusion in their culture media of some essential factor such as the yet unknown "pork factor" described here

The mechanism of the protection afforded by the avirulent diphtheria bacilli is noteworthy but not understood   Allergy apparently is not significant, for there is no enhanced skin reactivity to the *homologous* somatic antigen of the avirulent bacilli, but only to the heterologous antigen—the exotoxin of the virulent or ganisms   That resistance and survival are not due to the presence of antitoxin in the blood stream was pointed out by Frobisher and Parsons (1943) and was again demonstrated in these studies   Judging by the appearance of the local reaction and the relatively "bright" appearance of the test rabbits following the challenge dose, it would seem that there is some local tissue reaction which binds the toxin, delaying its general absorption or, in the case of the survivors, entirely preventing absorption by holding the toxin *in situ* until the animal has built up its own antitoxic (and possibly antibacterial) antibodies to combat the infection

In a general sense this is reminiscent of the observations by Abernethy and Francis (1937) that "some factor or change occurring in the serum in response to bacterial pneumococcal infection is capable of being mobilized in tissues and thereby reacting locally with the C substance" and that "the state of reactivity of the tissue cells is also essential for cutaneous response to C "

Whatever the nature of the phenomenon, it is obvious that some protection is afforded   In view of this fact, as well as of the mounting evidence that what is generally considered an adequate program of toxoid immunization is not always sufficient to prevent diphtheria (Eller and Frobisher, 1945, Turner, 1942), it seems permissible to suggest again that consideration be given to the idea that the immunizing agents used to protect children against diphtheria should contain properly cultivated bacterial antigens as well as antigens to stimulate anti exotoxin

SUMMARY AND CONCLUSIONS

Eighty-eight rabbits were repeatedly inoculated with living culture- of avirulent *Corynebacterium diphtheriae*   Twenty-two of the 88 animals received organisms which had been cultivated in a pork infusion medium   Of these 22

which was uniformly fatal to nonimmunized animals   The other 14 animals in this group of 22 survived an aveiage time of 5 3 days as contrasted to the 2 9-day average survival time of 31 control (nonimmunized) animals

This is in contrast with 66 rabbits which received inoculations of avirulent *C diphtheriae* cultivated on media not containing fresh pork   Of these 66 animals, none survived the challenge dose of virulent *C diphtheriae*, and their aveiage survival time of about 3 days was essentially the same as that (2 9 days) of the 31 control animals   Thiamine was shown not to be the essential antigen-adjuvant in the pork   The implications of these results have been discussed briefly with respect to immunization procedures in geneial, and especially those against diphtheria

Partial or complete protection against virulent diphtheria bacilli was engendered in rabbits by injecting into them living cultures of avirulent diphtheria bacilli which had been cultivated in a fresh pork base medium

Avirulent *C diphtheriae* cultivated in certain media not containing fresh pork were incapable of engendering any significant resistance against the virulent organisms

Fresh pork contains some factor which is critical for the antigenicity of the avirulent diphtheria bacilli under the conditions of these experiments   This factor is apparently not thiamine

The resistance of the immunized animals was not due to the presence of demonstrable antitoxin in the blood stream, and the mechanism of the protective action is not antitoxic   It appears to depend iather on a local binding action in subcutaneous tissues, wheie the unneutralized toxin causes extensive necrosis

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A STUDY OF BACTERIAL SYNERGISM WITH REFERENCE TO THE ETIOLOGY OF MALIGNANT DIPHTHERIA[1] [2]

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Malignant, "bull-neck," hypertoxic, or grave diphtheria is differentiated from "ordinary" or milder diphtheria by the presence of marked cervical swelling (hence "bull-neck"), extreme toxemia, albuminuria, frequent development of neuropathies, and a high death rate in spite of early administration of large doses of antitoxin Epidemics of diphtheria in which the malignant form predominate have been common in Europe, England, and elsewhere within the past two decades as reported by Anderson *et al* (1931) and Deicher and Agulnik (1927), but during the same period have been relatively uncommon on this continent Since 1931 only one such outbreak has been reported (Wheeler and Morton, 1942) However, in Baltimore, after several years of low morbidity and fatality, diphtheria began to increase in 1942 and, as in many other large cities in the United States and Europe during the war years, it attained high levels (Eller and Frobisher, 1945) During the first 6 months of 1944 there were in Baltimore 142 cases, 16 of which were designated as malignant The malignant cases suffered a 44 per cent mortality (7 cases), whereas the total mortality was only 6 per cent (9 cases) In 1945 there were 352 cases reported and 18 deaths A considerable number of these were malignant The disease continued at a relatively high level of incidence and severity throughout 1946

Since the description of gravis and mitis types of diphtheria bacilli by Anderson *et al* (1931), malignant diphtheria has been widely believed to be due to the gravis type of diphtheria bacilli, although it has been repeatedly pointed out by Frobisher (1943) and others that the occurrence of this organism, at least that variety of it which is found in Baltimore and elsewhere in the United States, bears no constant relation whatever to malignant diphtheria Continuous, systematic studies of the types of diphtheria bacilli found in cases of diphtheria in Baltimore since about 1932, including the numerous typical, fatal malignant cases noted above, have revealed during 16 years only 10 or 12 gravis strains, and these rarely in the malignant cases The mitis or mitis-like form has predominated in cases, contacts, and carriers at all times according to Frobisher (1938, 1940, 1942) This has also been found true, as a general rule, throughout the United States Obviously, then, malignant diphtheria in Baltimore and the United States during

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the period mentioned has depended on some other factor than the strains of gravis or mitis diphtheria bacilli found there

The possibility that a factor in malignant diphtheria might be the synergistic action of other bacteria has long been a subject of debate and experimentation Since the time of Roux and Yersin (1890) streptococci have most frequently been mentioned and investigated in this connection Unfortunately, studies prior to 1903 did not take into account the variability in virulence and toxigenicity of cultures of *Corynebacterium diphtheriae* Many workers did not use enough animals to give significance to their results None of them could identify their strains of streptococci with accuracy since they were working before the introduction of the bile solubility test for differentiating streptococci and pneumococci, the use of the blood agar plate, the method of differentiating streptococci on the basis of the type of hemolysis on blood agar, and the precipitin method of grouping beta hemolytic streptococci

More recent experimental studies on this problem have been conducted by Ramon and Djourichitch (1934) These authors concluded, in contrast with Roux and Yersin, that streptococci lower local tissue resistance to invasion by diphtheria bacilli but do not enhance the virulence of the latter Certain objections to these conclusions will be cited later

Dold (1927), Hopmann and Panhuysen (1931), Park and Williams (1933), and Stimson (1940) considered malignant diphtheria as due to combined infections Goepp (1938) and Cushing (1943) list several organisms that they believe may be involved These and most other authors stress the importance of streptococci The different views with regard to the role of streptococci and other factors in malignant diphtheria have been summarized previously (Frobisher, 1943)

Our own interest in streptococci as related to malignant diphtheria was kindled by the isolation of hemolytic streptococci of Lancefield group B from several successive cases of malignant diphtheria in Baltimore The diphtheria bacilli associated with these streptococci were all of the mitis type Subsequent experiments with these organisms in animals inclined us to the view that such streptococci are probably of etiological significance in malignant diphtheria Later studies of streptococci found in other cases of malignant diphtheria, however, failed to support this view, and left us unconvinced, one way or the other The experiments described below were therefore undertaken to investigate further the problem of bacterial associations in this disease

MATERIALS AND METHODS

Cultural procedures The cultural methods and media used were of a common type routinely employed in this laboratory and have been described elsewhere (Frobisher, 1938)

Bacteria Five strains of virulent *C diphtheriae* (nos C1 to C5) and two avirulent strains (C6 and C7) were used These are described in table 1

Five strains of beta type hemolytic streptococci isolated from the same patients as those yielding the correspondingly numbered virulent diphtheria strains

group

These pans of diphtheria bacilli and streptococcus cultures, each pair from the same patient, are called "homologous pans" or "homologous strains" in this report

One strain each of *Corynebacterium xerose* and *Corynebacterium pseudo-diphthericum* were isolated from normal persons

Other organisms Three strains of *Hemophilus influenzae*, type B, were obtained from the National Institute of Health [3]

Diphtheria toxin A well-ripened toxin (T106) produced by a Park Williams no 8 strain of *C diphtheriae*, and of tested potency, was used All dilutions of the toxin were made with Moloney and Taylor diluent prepared according to the

TABLE 1

STRAIN NO	TYPE	SOURCE	ASSOCIATED STREPTOCOCCI	
			Strain no	Lancefield group
C1	mitis	malignant diphtheria	S1	B
C2	mitis	malignant diphtheria	S2	B
C3	mitis	malignant diphtheria	S3	A
C4	minimus	malignant diphtheria	S4	B
C5	gravis	moderately severe diphtheria	S5*	A
C6	gravis avirulent	healthy carrier		
C7	gravis avirulent	healthy carrier		

* Streptococcus strain S5 came from a malignant case which was a contact of the C5 patient Both cases yielded a gravis type of diphtheria bacillus

formula in *Diagnostic Procedures and Reagents* of the American Public Health Association (1945)

Animal experiments Basically all of these experiments were alike, consisting in the injection of diphtheria bacilli or diphtheria toxin mixed with various other bacteria or substances into test animals The arrangement of controls was given particular attention, and each arrangement is described in detail in the appropriate place

EXPERIMENTS WITH MICE

White mice were selected as experimental animals because of their relatively great resistance to *C diphtheriae* and its toxin This resistance was considered advantageous for two reasons (1) Any synergistic reaction which might manifest itself would be the more obvious (2) Larger doses of diphtheria bacilli could be inoculated alone, for control purposes, or in combination with other organisms, without using antitoxin to prevent early deaths of the mice from diphtheritic

[3] Courtesy of Dr Margaret Pittman of the Biologics Control Laboratory, National Institute of Health

intoxication   In most experiments sublethal doses of diphtheria bacilli and streptococci were injected together intraperitoneally into 6 mice in a group The mixtures of organisms were made 15 minutes before injection

The results were compared with controls made up of similar groups of mice each receiving a dose of each organism alone equal to the largest amount of that organism in any tested combination   The proportions of one organism to the other varied in different experiments   Time and numbers of deaths were the criteria of severity of infection   A maximum observation period of 14 days was set arbitrarily   In order to minimize variables, age and weight limitations for the mice were adopted for most of the tests   It was found most convenient to use mice 6 to 9 weeks old weighing 18 to 23 grams   Closer limits were impracticable because of wartime shortages of mice   Cultures in broth, 48 hours old, were used routinely   It is to be noted, particularly in these first experiments, that, if a given dose of culture was to be less than 0 1 ml , the culture was diluted with sterile infusion broth so that the amount to be injected was 0 1 ml

The diphtheria bacilli and streptococci in these experiments were homologous pairs

PRELIMINARY EXPERIMENTS

Virulent C diphtheriae and beta hemolytic streptococci   A previously determined sublethal dose of each organism was inoculated into control groups of mice, and the same or smaller doses were combined for inoculation into the test groups In most cases this meant that the total volume of fluid inoculated into the test animals was greater, by 0 2 ml, than that given any of the controls   This was at first considered a negligible difference

Experiments of this type were conducted with the following homologous pairs of virulent *C diphtheriae* and hemolytic streptococci (beta type)   C1 and S1, C2 and S2, C3 and S3, C4 and S4, and C5 and S5   Protocols and results of two representative experiments are shown in table 2   In all instances the animals receiving a combination of virulent *C diphtheriae* and beta hemolytic streptococci of Lancefield group B died in much greater numbers, or more rapidly , or both, than those receiving either organism alone   The tentative inference drawn and reported at this time (Updyke and Frobisher, 1944) was that the reactions were evidence of a synergistic action between the two organisms

Experiments with hemolytic streptococci (beta type) plus avirulent C diphtheriae and nonspecific substances   Later, experiments were conducted with three strains of hemolytic streptococci (S1, S2, S4) in various combinations with (1) living and killed cultures of two strains of avirulent *C diphtheriae* (C6, C7), (2) sterile 10 per cent suspensions of animal charcoal and sterile 1 per cent suspensions of diatomaceous earth (pulverized Berkefeld filter) in infusion broth, and (3) sterile infusion broth   Typical results are shown in table 3

In general, deaths occurred in greater numbers, in a shorter time, or both, in the animals receiving the combinations of living streptococci and other substances *irrespective of the nature of the added substance   Deaths were in nearly direct proportion to the volume of the inoculum*

nutrient factor resulting from greater volume of broth and how much to the mechanical factors of size of inoculum and amount of particulate matter Whatever the explanation, it was evident that the results in the previously described preliminary experiments with virulent *C*

TABLE 2

Results of intraperitoneal inoculation into mice of virulent C diphtheriae and beta type hemolytic streptococci combined

RELATION OF CULTURES	ORGANISMS INOCULATED				MICE					
	Species	Strain no	Type or group	Amount (ml)	No inocu lated	Cumulative no dead (days)				Ratio of deaths to no inoculated
						1	4	7	14	
Control*	C diphtheriae	C1	mitis	0 2	6	0	0	0	0	0/6
Control*	S hemolyticus	S1	B	0 2	6	0	0	1	1	1/6
Combined	C diphtheriae	C1		0 2	6	2	4	6	6	6/6
	S hemolyticus	S1		0 2						
Combined	C diphtheriae	C1		0 2	6	1	4	6	6	6/6
	S hemolyticus	S1		0 1						
Combined	C diphtheriae	C1		0 1	6	0	1	2	4	4/6
	S hemolyticus	S1		0 2						
Control*	C diphtheriae	C2	mitis-like	0 3	6	0	0	0	0	0/6
Control*	S hemolyticus	S2	B	0 1	6	0	0	0	0	0/6
Combined	C diphtheriae	C2		0 3	6	4	4	5	6	6/6
	S hemolyticus	S2		0 1						
Combined	C diphtheriae	C2		0 3	6	4	5	5	5	5/6
	S hemolyticus	S2		0 05†						
Combined	C diphtheriae	C2		0 2	6	1	4	4	4	4/6
	S hemolyticus	S2		0 1						

* Culture alone   † 0 1 ml of a 1 2 dilution

diphtheriae were probably due to these factors rather than to a synergistic action between the diphtheria bacilli and streptococci

   Further experiments with virulent and avirulent C diphtheriae, hemolytic strepto-cocci (beta type), and nonspecific substances   In these experiments the combined action of homologous pairs of virulent diphtheria bacilli and streptococci was again studied, but in these tests there were included control groups of mice receiving each organism in combination with avirulent diphtheria bacilli (C6), inert particles, or infusion broth   An acute war-induced shortage of mice limited these fully controlled experiments to three two with organisms C1 and

S1, and one with C2 and S2   In each experiment a total of 15 to 16 control and

TABLE 3

Results of intraperitoneal inoculation in mice of beta type hemolytic streptococci combined with suspensions of living and killed avirulent C diphtheriae, charcoal, diatomaceous earth, and sterile infusion broth

RELATION OF MATERIALS	MATERIALS INOCULATED				MICE					
	Species and substances	Strain number	Type or group	Amount (ml)	No inoculated	Cumulative no dead (days)				Ratio of deaths to no inoculated
						1	4	7	14	
Control*	C diphtheriae	C6	gravis	0 8	6	0	0	0	0	0/6
Control*	S hemolyticus	S1	B	0 2	6	0	1	1	1	1/6
Combined	C diphtheriae	C6	gravis	0 8	6	6	6	6	6	6/6
	S hemolyticus	S1	B	0 2						
Combined	C diphtheriae	C6	gravis	0 6	5	4	4	4	4	4/5
	S hemolyticus	S1	B	0 2						
Control*	C diphtheriae	C7	mitis	0 7	6	2	2	2	2	2/6
Control*	C diphtheriae	C7	mitis†	0 7	6	0	0	0	0	0/6
Control*	S hemolyticus	S4	B	0 025‡	6	0	5	5	5	5/6
Control*	S hemolyticus	S4	B†	0 025‡	6	0	0	0	0	0/6
Combined	C diphtheriae	C7	mitis	0 7	6	6	6	6	6	6/6
	S hemolyticus	S4	B	0 025‡						
Combined	C diphtheriae	C7	mitis†	0 7	6	6	6	6	6	6/6
	S hemolyticus	S4	B	0 025‡						
Combined	C diphtheriae	C7	mitis	0 7	6	0	0	0	1	1/6
	S hemolyticus	S4	B†	0 025‡						
Control*	S hemolyticus	S4	B	0 025‡	6	0	2	2	2	2/6
Combined	Charcoal 10%§			0 1	6	1	5	6	6	6/6
	S hemolyticus	S4	B	0 025‡						
Combined	Berkefeld filter 1%§			0 2	6	0	4	4	4	4/6
	S hemolyticus	S4	B	0 025‡						
Combined	Infusion broth			0 8	6	6	6	6	6	6/6
	S hemolyticus	S4	B	0 025‡						

* Culture alone   ‡ 0 1 ml of a 1 4 dilution
† Killed   56 C waterbath 75 min   § Suspended in infusion broth

test groups of mice were inoculated within 2 to 3 hours with cultures from the same sources

RELATION OF MATERIALS	MATERIALS INOCULATED					MICE					
	Organisms or nonspecific substance	Strain no	Type or group	Virulence	Amount (ml)	No inoculated	Cumulative no dead (days)				Ratio of deaths to no inoculated
							1	4	7	14	
Control*	*C diphtheriae*	C1	mitis	+	0 2	12	0	0	0	1	1/12
Control*	*C diphtheriae*	C6	gravis	−	0 6	12	0	0	0	0	0/12
Control*	*S hemolyticus*	S1	B		0 1	12	0	0	0	0	0/12
Combined	*C diphtheriae* Infusion broth	C1	mitis	+	0 2 / 0 1	6	0	0	0	0	0/6
Combined	*C diphtheriae* Charcoal 10%†	C1	mitis	+	0 2 / 0 1	12	0	0	0	0	0/12
Combined	*C diphtheriae* Infusion broth	C1	mitis	+	0 2 / 0 6	12	0	0	0	0	0/12
Combined	*C diphtheriae* Charcoal 10%†	C1	mitis	+	0 2 / 0 6	12	0	0	0	0	0/12
Combined	*C diphtheriae* *C diphtheriae*	C1 C6	mitis gravis	+ −	0 2 0 6	12	0	2	4	5	5/12
Combined	*C diphtheriae* Infusion broth	C6	gravis	−	0 6 0 2	12	0	0	0	0	0/12
Combined	*C diphtheriae* Charcoal 10%†	C6	gravis	−	0 6 0 2	12	0	0	0	0	0/12
Combined	*S hemolyticus* Infusion broth	S1	B		0 1 0 2	12	0	1	1	2	2/12
Combined	*S hemolyticus* Charcoal 10%†	S1	B		0 1 0 2	12	3	3	3	3	3/12
Combined	*S hemolyticus* *C diphtheriae*	S1 C1	B mitis	+	0 1 0 2	12	0	2	2	3	3/12
Combined	*S hemolyticus* Infusion broth	S1	B		0 1 0 6	12	4	6	6	8	8/12
Combined	*S hemolyticus* Charcoal 10%†	S1	B		0 1 0 6	12	10	12	12	12	12/12
Combined	*S hemolyticus* *C diphtheriae*	S1 C6	B gravis	−	0 1 0 6	12	7	9	11	11	11/12

* Culture alone   † Suspended in infusion broth

The results obtained with both pairs of organisms (C1 + S1, C2 + S2) were closely parallel   Representative data are shown in a composite table of the two tests run with C1 and S1 (table 4)   The combination of the streptococcus (S1) with equal amounts (0 2 ml) of virulent *C diphtheriae* (C1), 10 per cent charcoal, or infusion broth resulted in a definite, though small, increase in deaths as compared with controls   3, 3, and 2, respectively, among 12 animals   Only 1 of the *C diphtheriae* and none of the streptococcus control animals died   Combination of streptococci with larger amounts (0 8 ml) of any agent, whether avirulent *C diphtheriae*, 10 per cent charcoal suspension, or sterile infusion broth, resulted in a much more marked increase in mouse deaths   11, 12, and 8, respectively, among groups of 12 animals

In contrast with the above-described results obtained by combining various agents with streptococci, the combining of inert particles, infusion broth, and other agents with virulent and avirulent diphtheria bacilli did not alter the results, i e , all the mice survived [4]

DISCUSSION

The results of our first experiments with streptococci were thought to indicate a synergistic interaction between certain strains of diphtheria bacilli and beta hemolytic streptococci   In later experiments, however, similar results were obtained with three strains of streptococci in combination with nonspecific substances   It is probable, therefore, that all the results involving streptococci were a manifestation of the influence of volume of fluid, pabulum, or particulate matter on these organisms (or on host resistance) and that no synergism occurred in any of the tests   In contrast with streptococci, the strains of *C diphtheriae* used in this study exhibited no enhancement of lethal effect as a result of similar combinations

In view of these results and of the observations of Djamil (1934) regarding the importance of the quality of the suspending fluid in such experiments, the role of the streptococci in the reactions reported by Ramon and Djourichitch (1934), and by ourselves, is debatable   The latter workers first determined sublethal doses of saline suspensions of *C diphtheriae* and then inoculated guinea pigs with such suspensions in combination with streptococcus cultures in broth or in filtrates of these   It seems probable that they would have obtained similar results with combinations of the streptococcal suspensions and sterile broth   Similar errors probably existed in many of the early investigations

In conclusion of this discussion on the experiments with mice, it may be said that under these experimental conditions—

(1) The lethality of some strains of beta hemolytic streptococci for mice L

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[4] In one test, for an unexplained reason, 5 of 6 mice died after receiving the virulent avirulent diphtheria bacilli combination (C1 + C6), but all 6 animals survived the corresponding inoculation in the parallel test with C2 and C6   Also, the virulent avirulent diphtheria bacillus combination (C2 and C7) was not lethal to the mice   Unfortunately, no more mice were available for further study

·em, and

(2) The increased lethal effect observed when *C diphtheriae* and beta type hemolytic streptococci are combined can best be explained as due to the increased volume of nutrient material   In view of this, the increased lethality observed in mice in these experiments cannot be attributed to a true synergistic action between the two organisms

It was necessary to discontinue the line of investigation just described because of a critical shortage of mice arising from greatly increased military demands Since the supply of guinea pigs was also limited, the investigation was continued employing rabbits as experimental animals   Their use necessitated a considerable modification in technique because of the extreme susceptibility of rabbits to diphtheria infection and intoxication   Modifications were also made to eliminate error due to uncontrolled variations in the volume and nature of inoculum such as those which had proved to be so important in the experiments with mice   In addition, the study was expanded to include organisms other than streptococci

In general, 3 rabbits were used in parallel for each test, all 3 receiving the same control and combined inocula   Inoculations were made intracutaneously in the dorsal skin of rabbits which had been shaved with electric clippers   The relative position of control and combined inoculations were the same for all three animals in a single test but were varied from one test to another to eliminate the possibility of differences in local tissue response   In all injections the volume of the inoculum was 0 2 ml   The quality of the fluid varied in accordance with the nutritive requirements of the organisms under study   Observations on the animals were continued until all reactions were subsiding (usually 2 to 4 days)

Virulent C diphtheriae and hemolytic streptococci (beta type)   A few tests were carried out with living cultures of virulent *C diphtheriae* and streptococci, Lancefield group B (C1 and S1), alone, in combination with each other, and in combination with 10 per cent animal charcoal in infusion broth   Four hours after the test injections the animals were given 1,000 units of diphtheria antitoxin intravenously in order to prevent early deaths from diphtheritic intoxications   Immediately after the administration of antitoxin, duplicate inoculations in adjacent sites were made of all the test materials to determine whether the combined infections were antitoxin-refractory

The following results were obtained in these tests

(1) The areas of erythema and necrosis produced by the combinations of diphtheria bacilli and streptococci inoculated before administering antitoxin were in some cases definitely, but not markedly, larger than those produced by either organism alone

(2) The progress of the diphtheritic infections, whether alone or in combination with the streptococci, *was promptly arrested by antitoxin*

(3) The addition of charcoal suspension to either the streptococci or the diphtheria bacilli did not result in an enhanced reaction

USE OF TOXIN INSTEAD OF C DIPHTHERIAE

Since the infections were controlled by antitoxin, which in itself does not inhibit the growth of diphtheria bacilli, it seemed probable that the most important factor in the enhanced reactions mentioned in conclusion 1, above, was the exotoxin which presumably was formed and fixed in the tissues *before* the antitoxin was administered   Therefore, it seemed possible that the antitoxin, by its neutralization of toxin produced *after* its administration, had arrested all the reactions, since these would depend for their further evolution on continued elaboration of free toxin by organisms growing in the tissues   This would spoil the experiments by killing the animals quickly   To eliminate this possibility a technique was developed which did not require the use of diphtheria organisms or antitoxin, e g , cultures of *C diphtheriae* were replaced by sterile toxin Since the identity of toxins from all strains of *C diphtheriae* appears to have been demonstrated by Parish *et al* (1932), Povitsky *et al* (1933), Zinnemann and Zinnemann (1939), and Zinnemann (1946), the substitution of PW no 8 toxin for that produced *in vivo* by other strains of *C diphtheriae* (C1, C2, etc) seems to need no special justification   It was decided to use a constant amount of toxin despite the fact that the experimental conditions were thereby made less like a normal synergistic association of two living organisms

The use of a standard toxin dosage was advantageous in that it permitted careful control of this factor   It was disadvantageous in that it provided a fixed amount of toxin in the tissue at one time, whereas the living diphtheria cultures supplied continuous small amounts, although when the diphtheria cultures were used there was an unknown and variable amount of toxin taking part in the reaction

The toxin (T106) was diluted so that 0 1 ml, the dose used, contained just enough toxin to cause a slight necrosis on intracutaneous inoculation in normal rabbits

The materials for injection were as follows

(1) *Toxin control*   Toxin dilution plus a volume of the nutrient base (plain or blood infusion broth) equal to that in which the particular dose or organism under study was suspended

(2) *Organism control*   Culture plus an equal volume of Moloney and Taylo diluent (1932)   The toxin was diluted in Moloney and Taylor diluent

(3) *Combined*   Toxin dilution plus an equal volume of culture[5]   All ino ul were equal in volume

[5] The plain or blood infusion broth in the toxin control and the Moloney and Tayl diluent in the organism controls equalized the volumes of these substances in the c ~ bination   The undiluted toxin was in an infusion broth base and, therefore, the dilute toxin preparation contained infusion broth diluted with Moloney and Taylor diluent to approximately 1 in 200   This small amount of nutrient was disregarded in the prepa s tion of the culture controls

With most of the test organisms the reactions produced in combination with diphtheria toxin were regularly identical with, or only slightly different from, those produced by the organism or toxin alone [6] With some of the test organisms, the areas of erythema, edema, and necrosis produced by the combination with diphtheria toxin were sometimes larger than those produced by either alone [7]

The tests with *Diplococcus pneumoniae* type I were especially interesting In three animals inoculated intradermally in the manner described above, enormous areas of erythema and very marked and extensive edema developed at the site of inoculation of the combination of the diphtheria toxin and pneumococcus culture, but not at either control site One of the three animals died in 3 days However, similar extensive reactions (with occasional deaths) developed in some rabbits at the pneumococcus control site and not at the toxin pneumococcus site In three animals inoculated with heated[8] toxin and pneumococcus cultures similar results were obtained extensive reactions developed in one animal at the pneumococcus control site only, in another at the toxin pneumococcus site only, and in the third at both the pneumococcus control and toxin pneumococcus sites Finally, to dissociate the toxin from the phenomenon entirely 13 rabbits were inoculated with a pneumococcus culture alone Three of these 13 animals developed the typical extensive reaction, and 1 died in 7 days Only small abscesses developed in the other rabbits These observations are in accord with those of Goodner (1928) and of Abernethy (1937) on the effect of type I pneumococci on rabbits

In view of the fact that in some cases an extensive reaction originated from one pneumococcus inoculation and not from another in the same animal, it seems probable that the difference between the various responses was due solely to variations in local tissue resistance, minor alterations in injection technique, or both

DISCUSSION

In this section of the investigation 32 different species of bacteria were injected into rabbits in combination with diphtheria toxin The association of 19 of these species with the toxin did not result in any significant difference between the reactions produced by the tested combinations and the reactions produced by the organisms or toxin alone With 13 of the organisms, the

[6] *Hemolytic streptococci* (beta type), Lancefield groups A and B, *streptococcus* (alpha type), streptococcus (gamma type), *H influenzae* (type unknown), avirulent *C diphtheriae*, gravis and mitis types, *C pseudodiphthericus*, *D pneumoniae*, types I and II, *N catarrhalis*, *N intracellularis*, *N gonorrhoeae*, an unidentified, gram positive, biscuit-shaped diplococcus from a throat culture, and a species of *Lactobacillus* from the trochea of a fatal can of malignant diphtheria

[7] *Streptococcus* (beta type), groups B and G, *S aureus*, *H influenzae*, type B, *C xerose*, *Klebsiella* sp , *E coli*, *E typhosa*, *N sicca*, and *D pneumoniae*, type III

[8] At 70 C for 10 minutes

reactions at the sites of inoculation of the combined preparations were larger in one or more of the rabbits than those produced by the toxin alone or the organism alone   These occurred so irregularly that their significance with respect to synergism seems doubtful

There was no correlation between the type, species, or genus of the organism, and the property of developing enhanced reactions in association with diphtheria toxin   Organisms from several entirely unrelated genera exhibited the property, but not all species of the same genus, or even types of the same species, did so   For example, enhanced reactions occurred with 1 or more species of *Streptococcus, Hemophilus, Escherichia,* etc , but with only 1 of 4 species of *Neisseria* and with only 2 of 3 strains of hemolytic streptococci (beta type), group B

The studies with one strain of pneumococcus type I indicated that variations in host resistance or in local tissue resistance, slight alterations in injecting technique, or all three, were responsible for marked differences in the reactions produced by those organisms   It seems likely that most of the differences in the tests with other bacteria were due to the same factors   The possibility is not denied, however, that the association of the toxin with some of these organisms may have been in some way responsible for the development of enhanced reactions

Since this study was initiated to investigate bacterial synergism with regard especially to malignant diphtheria, it is interesting to consider the possible significance of the reactions described above in relation to that disease   The ratio of severity of reaction produced by the toxin or organisms alone to those produced by combinations was in no wise comparable to the ratio of severity of ordinary diphtheria to that of malignant diphtheria   Furthermore, in the experiments in which antitoxin was administered before the test injections were given, the enhanced reactions did not develop, indicating complete control of the diphtheritic intoxication, whereas malignant diphtheria is characteristically antitoxin-refractory   In view of these facts and the variety and number of organisms which exhibited the enhanced reaction with diphtheria toxin, it seems unlikely that synergism has any great significance in malignant diphtheria

It is recognized, however, that conditions prevailing in the human disease are different from, and probably more complex than, those in these experiments In the first place, the differences in the host and the site of infection preclude any direct comparison, and, in the second place, most of the experiments were carried out with a fixed amount of toxin, whereas in the human disease there are living diphtheria bacilli producing a continuous supply of small amount of toxin   Therefore, though the possibility of a significant degree of synergistic action between diphtheria bacilli (or toxin) and other organisms in human infections is not eliminated, the evidence obtained from the experiments herein described in no way supports it

SUMMARY AND CONCLUSIONS

The problem of the etiology of malignant diphtheria was investigated with reference to the possibility of a synergistic action between *Corynebac*-

binations of living cultures of homologous pairs (both organisms isolated from the same case of malignant diphtheria) of *C diphtheriae* and streptococci (beta type), and (2) intracutaneous inoculation, into rabbits, of diphtheria bacilli or toxin in combination with living cultures of various organisms

In the experiments with mice deaths occurred in greater numbers and in a shorter time among those animals receiving combinations of sublethal doses of diphtheria bacilli and streptococci than among those receiving the same dose of either organism alone   This was at first interpreted as indicative of a synergistic action between the two organisms   However, similar results were obtained with three of the same strains of streptococci when sterile infusion broth, nonspecific particulate matter, or both were substituted for the cultures of diphtheria bacilli   It was concluded that the volume or quality of nutrient material or inert particles, or both, in the inoculum has a marked effect on the lethality of streptococci for mice   In three experiments in which these factors were adequately controlled no synergism was apparent between two homologous pairs of *C diphtheriae* and hemolytic streptococci (beta type), group B   It is probable that the factors of pabulum and particulate matter were responsible for all the results and that no synergism occurred between any of the strains of *C diphtheriae* and streptococci used

The experiments with rabbits in which nonspecific factors in the inocula were controlled revealed no reactions that could definitely be attributed to synergism between diphtheria toxin and one or more strains of 32 different bacteria

No clear-cut evidence has been obtained of a true synergism between *C diphtheriae*, or its toxin, and a variety of other organisms

Interpretation of the results of experiments on bacterial synergism must be made with due regard to the influence of nonspecific factors, which were found to modify significantly the apparent virulence of some of the organisms used in this investigation

The problem of the etiology of malignant diphtheria is as yet unsolved Further investigations are essential to determine whether the factors of etiological significance are related to the diphtheria bacillus or its products, to host factors, or to bacterial synergism

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...NE(5)SULFONATE
ON A STRAIN OF EBERTHELLA TYPHOSA

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In recent years acenaphthene has been found to cause polyploidy in plants Shmuck (1938), Kostoff (1938a, 1938b), Navaskin (1938), and Shmuck and Gusseva (1939) have indicated that acenaphthene may be superior to colchicine for inducing mutations in higher plants   Ark (1946) reported that induced, permanent mutations were formed in broth saturated with acenaphthene in *Phytomonas michiganensis* and *Erwinia carotovora*   Luria (1947) reports, however, that Ark's results suggest selection for mutants rather than induction of mutations   The present report is concerned with the effect of an isomeric sulfonate of acenaphthene on a strain of *Eberthella typhosa*   This aromatic compound is sodium acenaphthene(5)sulfonate

METHODS AND RESULTS

The strain of *Eberthella typhosa* used in this study had been isolated from the blood of a typhoid patient   A single cell of this culture was isolated and put into nutrient broth for a previous study concerning the effects of X-rays on this strain (Grainger, 1947)   This single cell isolation of this culture was used for this study as well   The strain was characteristic of the species in respect to all the biochemical and physiological characteristics as described in Bergey's *Manual* (1939)   Neither its antigenic formula nor its phage specificity was determined

The culture was subcultured daily into nutrient broth as well as plated on nutrient agar by the streak method for 14 successive days before being used in the study   Observations were made on the colonial character after 24-hour incubation on nutrient agar   The colonies were studied by means of a colony microscope lens (3 ✕) to note any change in morphology   The colony character of the strain remained a constant smooth type

A 2 per cent aqueous solution of sodium acenaphthene(5)sulfonate[1] was made and sterilized   The final reaction was pH 6 8   Various amounts of this solution were added to 10 ml of nutrient broth (pH 7 2) to determine the amount needed to inhibit the growth of this strain of *Eberthella typhosa*   One loopful (4 mm) of a 24-hour nutrient broth culture was added to each tube containing the different concentrations of the compound in nutrient broth   It was found that as high as 5 ml of the 2 per cent solution in 10 ml of nutrient broth failed to inhibit the growth of the strain of *Eberthella typhosa*

It was of interest to note whether sodium acenaphthene(5)sulfonate would have any effect on the colonial character of this culture   Thus, to a flask containing

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[1] The sodium acenaphthene(5)sulfonate was kindly supplied by Prof R T Wendland, Chemistry Department, Lehigh University

95 ml of sterile nutrient broth were added 5 ml of a sterile, aqueous, 2 per ce_ solution of this compound   This amount of the compound in the nutrient bro'h was 0 1 per cent   The final pH was 7 0   One loopful of the 24-hour nutr·r' broth culture of *Eberthella typhosa* was added to this flask   A loopful was hk wise added to a flask containing 100 ml of nutrient broth   This served a. s control   The flasks were placed in the incubator at 37 C

Subcultures on nutrient agar plates by the streak method were made daily fc, 30 days from the flask containing the compound in the nutrient broth as well a from the control nutrient broth   The colonies were studied by means of a colour microscope (3 ✕) to note any changes in morphology   At least 100 well i olated colonies were studied daily on the nutrient agar obtained from the subcultun from each flask

The colonies from the flask of nutrient broth containing the sodium acenap^ thene(5)sulfonate showed no changes until the seventh day   At this time, 1" colonies out of 100 examined were of the R type   From this time on the daily subcultures showed a percentage of rough forms that varied between 2 and 1' The percentage of the R type varied from day to day, but this was probably du-' to the chance in isolating the rough forms which had appeared on the seventh day   The R type did not displace the original S type   The period of obsena tion ended at 30 days

The R colony of *Eberthella typhosa* was picked and placed in nutrient broth fo further study   It was found to be characteristic of the species in respect to all the biochemical and physiological characteristics as described in Bergey ' *Manual* (1939), except for one difference   it would not ferment the mono ac charide, galactose   The R colony character of the strain remained constant afte repeated subcultures over a period of 3 months

There was little variation in the colonies from the control nutrient broth Occasionally an intermediate colony was observed   All the other colonr showed a typical smooth type of colony   This observation has been noted wi'h this culture for a period of over 6 months

Since it is desirable to avoid any implication concerning the hereditary mecha nism of bacteria until we have more adequate knowledge, the term *anomal* ' *variation* has been suggested by Grainger (1947)   The modification of this cul ture by the use of this compound is an anomalous variation, as well as any o'l variation the causative mechanism of which is unexplained at the present tim- It is possible that the anomalous variation that occurred in this study may I been spontaneous and that the environment favored the growth of the re r'1 organisms to this compound   It has been shown repeatedly, for examplo, the bactericidal and bacteriostatic substances act as selecting agents to permi' t' detection of resistant strains   As Luria (1947) has warned, "One should t particularly cautious before claiming induction of mutation by environr---' agents when the change appears to affect the whole population expo-ed   I very likely that in such cases a type arisen by spontaneous mutation ha c ~ pletely displaced the original type because of favorable selection by the o ~ environment "

ı ı " variation The substance did not appear to be bacteriostatic in the concentration used and the R type that appeared on the seventh day did not displace the original S type The percentage of rough colonies did not seem to increase from the time they first made their appearance until the period of observation ended 23 days later Thus, it would seem that the anomalous variation that occurred was due either to the effects of this compound or merely to a spontaneous change

SUMMARY

One-tenth of a per cent of sodium acenaphthene(5)sulfonate in nutrient broth produced an anomalous variation from a smooth culture of *Eberthella typhosa* A rough type colony was first observed after the seventh day of incubation The percentage of rough colonies varied daily between 2 and 18, from this period until the observations ended at 30 days The rough type did not displace the original S culture

The rough strain of *Eberthella typhosa* was found to be characteristic of the parent S strain in respect to all the biochemical and physiological characteristics, except that it did not ferment galactose

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Thus in the unshadowed group three measured 710 mμ, one 740 mμ, and one 770 mμ   In the shadowed group one measured 780 mμ, one 800 mμ, two 830 mμ, one 840 mμ, one 860 mμ, and two 920 mμ   Representative examples are shown in figure 1, nos 1 to 4

It is believed that these bodies lie so far on the high side of the range of the distribution of diameter, shown to be normal, as to be significant   They certainly would appear larger in the light microscope than the commonly accepted elementary bodies and would thus fill the criterion for initial bodies   It is true they are few in number—only 0 5 per cent in the present series—but this proportion is certainly of the same order of magnitude as the proportion of initial bodies making up the viral suspensions prior to drying them on the collodion membrane

As has been pointed out above, previous studies (Rake and Jones, 1942) had indicated that the so-called plaques represented colonies of elementary bodies embedded in a "capsular" matrix   Examination of the series of electron micrographs of the agent of feline pneumonitis showed groups of bodies which could be interpreted as colonies, since the close juxtaposition of the bodies and the molding together of their contiguous surfaces would favor such an hypothesis rather than one of secondary agglutination (figure 1, nos 5 and 6)   It is true that no surrounding "capsular" matrix was to be observed, but this is not surprising   As has been shown elsewhere (Rake and Jones, 1942), smear preparations even from yolk cells shown by section to be loaded with plaques never show any such plaques in an intact state, even with the light microscope, and fragmenting plaques are rare   If this is the case with the more gentle techniques involved in preparation for examination under the light microscope, the failure to demonstrate any intact "capsular" matrix in the present preparations is not surprising

SUMMARY

Examination of electron micrographs of the agent of feline pneumonitis has demonstrated the existence of large bodies lying well outside the range of size found for the elementary bodies   Such large bodies form approximately 0 5 per cent of all bodies studied   They are believed to represent initial bodies   It is also possible to demonstrate closely packed groups of elementary bodies which are presumed to represent the colonies of elementary bodies usually found in sections of infected yolk sac embedded in a "capsular" matrix to form the

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No 1 Elementary bodies and one initial body not shadowed with gold   14,220
No 2 Elementary bodies and two initial bodies not shadowed with gold   14,450
No 3 Elementary bodies and one initial body, gold shadowed, 21 7 mg of gold, angle 11°32', 10 cm distance   14,455 ×
No 4 Replicas of one elementary and one initial body, gold shadowed, 1.5 mg of gold, angle 18°26', 9 cm distance   14,140 ×
No 5 Small group of elementary bodies, gold-shadowed, 21 7 mg of gold, angle 11°, 10-cm distance   14,220 ×
No 6 Two groups of virus particles, gold-shadowed, 25 2 mg of gold, angle 10°, 10 cm distance   14,500 ×

1

2

3
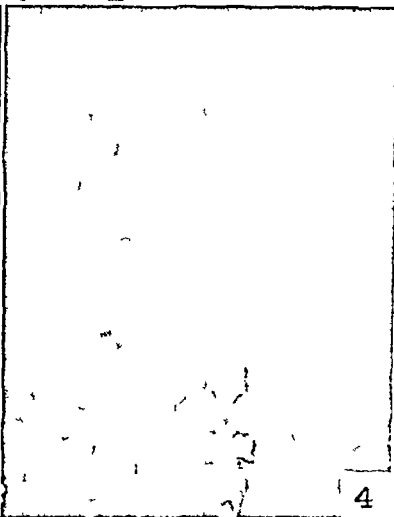
4
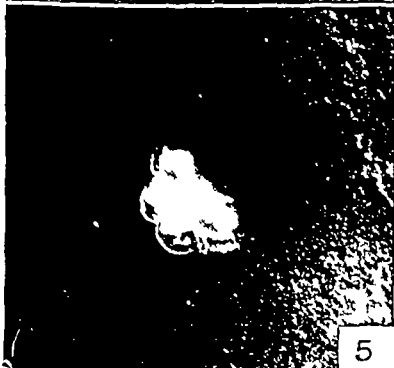
5
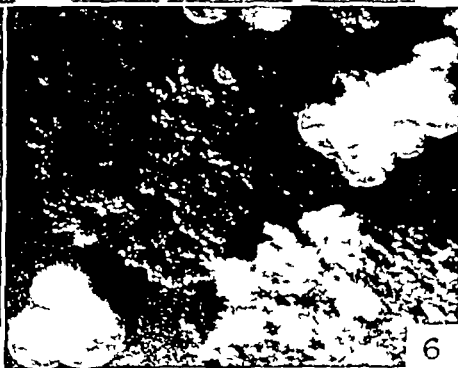
6

plaque It is believed that the matrix itself is easily disintegrated and so d appears during preparation of the screens for the electron microscope, a th... in accord with other observations

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THE RELATIVE ERRORS OF BACTERIOLOGICAL PLATE COUNTING METHODS

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It is generally conceded that the degree of precision ordinarily attained with dilution plate counting methods of enumerating viable bacteria is not so great as could be desired   The extent of variation encountered even under the best conditions is considerably larger than the maximum permitted, for example, of routine chemical analyses   Coefficients of variation consistently as high as 30 per cent of the mean have been reported (Mudge and Lawler, 1928, Ziegler and Halvorson, 1935), although considerably lower values have also been encountered (Jennison, 1937)   Some of the principal sources of variation which may be identified at the present time are

(1) The presence of clumps, consisting of various numbers of organisms, in the original suspension (Jennison, 1937)

(2) The error involved in preparing a dilute suspension (Jennison and Wadsworth, 1940)

(3) The error involved in measuring aliquots of this suspension into plates

(4) The distribution of organisms in these aliquots (Fisher, Thornton, and Mackenzie, 1922, James and Sutherland, 1939, Sutherland and James, 1938, Wilson and Kullmann, 1931)

(5) Factors which influence the development of the inoculated cells into visible colonies (Harmsen and Verweel, 1936–1937, McNew, 1938)

The relative importance of these different sources of error depends considerably upon the conditions of experimentation, but it appears probable that if species of bacteria characterized by persistent or tenacious clumping are excluded, the contribution of the first two sources listed above may be reduced through careful technique to a small part of the total   It is not an uncommon practice to describe the variability of replicate plates as the total error of the plate count, and this is probably not often a serious misrepresentation   However, methods of estimating "dilution error" have been described (Jennison and Wadsworth, 1940) and are undoubtedly valuable for precise studies

The sum of the variations due to the three remaining sources may be estimated from the observed variability of replicate plates   The chief purpose of the present study is an analysis of this sum, separating the variation related to the measurement of aliquots into plates from the sum of the remaining two factors, and estimating its relative importance   The data required for this purpose were obtained by comparing several measuring methods with respect to the volumes of suspension delivered and the plate counts obtained with each method   This problem does not appear to have been investigated specifically, although methods for increasing the precision of measurement have been devised and recommended

for use (Miles, Misra, and Irwin, 1938, Wilson, 1922)   Since the comparison was also designed to assist in the selection of appropriate bacterial counting methods for practical use, several modifications, which are not strictly essential to the principal objective, were included and assessed

METHODS

Three methods of measuring aliquots of inoculum were employed, and these, together with other modifications of technique, provided five plating methods for comparison, as follows

A   Prepared and dried plates were inoculated with 6 drops of a known dilution of organisms delivered by means of a single calibrated capillary pipette [1] The drops were allowed to fall at well-separated points on the surface of the agar

(1) The area covered by the individual drops was increased by repeatedly tilting the plate in all directions   This method is commonly used with *Brucella* species

(2) The inoculum was distributed over the entire surface of the plate with a sterile, bent glass rod

B   Prepared and dried plates were inoculated with 0 1-ml quantities of dilute suspension delivered by means of a single "exax" serological 1-ml pipette gradu ated in tenths and hundredths of a ml   The inoculum was distributed by means of a sterile, bent glass rod   This method has been used routinely with *Bacterium tularense* (Snyder, Engley, Penfield, and Creasy, 1946) and is a modification of the method of Anderson and Stuart (1935)

C   Nine-tenths ml of dilute suspension were delivered by means of a single "exax" 1-ml serological pipette into the following

(1) Sterile, empty plates, which then received 15 ml melted agar medium held at 45 C   The medium and inoculum were mixed and allowed to solidify   This is the conventional poured plate, with the single exception that an attempt was made to increase accuracy by avoiding delivery of the final 0 1 ml of the inoculum

(2) Culture tubes (16 by 150 mm) containing 2 ml of melted 3/2 strength agar medium held at 45 C   The medium and inoculum were mixed and allowed to solidify on the walls of the tube by rotating the tube in a horizontal position under the cold water tap   This is the rolled tube method (see Wilson, 1922) modified with respect to the size of the inoculum

In order to provide a valid basis for comparison, a single bacterial species was counted with all five methods   *Shigella dysenteriae*, strain 14-4, was selected for this purpose because its rather simple nutritional requirements and its relative indifference to oxidation-reduction potential could be expected to permit abun dant growth in all cases

Final dilutions for plating were prepared from 24-hour stock cultures in tubes of nutrient broth, using as the diluent 0 2 per cent Difco gelatin in 1 0 per cent disodium phosphate (12H$_2$O) adjusted to pH 6 8   Whenever the same dilu tion

[1] We are indebted to M/Sgt D E Drukenmiller, Jr , for a supply of those pi which were prepared essentially according to the directions of Donald (1915)

bottle of that dilution

Tryptone agar of the following composition was used in all plates

Difco tryptone	2 0 per cent
Sodium chloride	0 5 per cent
Glucose	0 5 per cent
Difco agar	2 0 per cent
Adjusted to pH 7 0 to 7 2	

In the case of method C2 (rolled tubes), all ingredients were originally present in concentrations 1 5 times those indicated, but the addition of the inoculum reduced them to approximately those listed

All surface-inoculated plates were dried before being used by storage at 34 C for 3 to 5 days   This is not recommended as a method for producing uniformly dried plates, but it is believed that the extent of drying is a relatively unimportant factor in the case of *S dysenteriae*

Plates and tubes were incubated at 34 C for 24 hours after inoculation   Plates were counted with the aid of a Quebec colony counter, whereas rolled tubes were counted over oblique illumination, each colony being marked with a wax pencil Counting rolled tubes was found to be extremely tedious

The volumes of fluid delivered by means of the three pipetting methods were determined gravimetrically   Approximately 60 replicates were delivered into weighed sample bottles by each method, the exact technique used in plating being followed as nearly as possible   The bottles were closed immediately after the addition and weighed to the nearest one-tenth of a milligram   However, in order to reduce the total number of weighings required, distilled water was substituted in these measurements for gelatin-phosphate diluent, so that the bottles might be merely dried, rather than cleaned and reweighed, before being used again This method appeared to be valid, since the difference in weight of the dry sample bottles before and after completion of all measurements averaged only $+0 7$ mg, and in only one case (apparently an error in the initial weighing) did it exceed 2 0 mg, or 2 per cent, of the smallest volume measured

The substitution of distilled water necessitated a correction for the volume of gelatin-phosphate diluent delivered by the capillary-dropping pipette method (A), since the volumes of the drops formed are influenced by the surface tension of the fluid delivered   The correction factor required was determined by counting the number of drops of distilled water and diluent delivered by the particular capillary pipette used throughout   The pipette was mounted vertically in a fixed position, and the drop rate was controlled by means of a capillary air inlet A constant volume was assured by horizontal markings on the pipette, and the portion of the last drop delivered was estimated to the nearest one-tenth of a drop   The results were quite constant with water, averaging for 11 determinations 102 05 $\pm$ 0 05 drops   They were equally constant for any one sample of gelatin-phosphate diluent, but different samples differed appreciably the average of 5 determinations on each of 6 samples was 105 56 $\pm$ 0 18 drops   The differ-

ence between water and diluent is highly significant   On the basis of the averages the ratio of volume of drops of diluent to volume of an equivalent number of drops of water is 0 96675   This is considerably less than the theoretical cal ratio calculated from surface tension measurements and probably may be accounted for by the small diameter of the pipette tip

<div align="center">STATISTICAL METHODS</div>

In order to compare the magnitude of plate counts obtained by different methods of plating, the observed mean counts were adjusted according to the dilution and volume of suspension inoculated   In all cases the adjusted mean count is that expected with 0 1 ml of the 10^{-5} dilution

Standard deviations,

$$s = \sqrt{\frac{\Sigma(x - \bar{x})^2}{N - 1}}$$

(x = counts of individual plates, $\bar{x}$ = mean count, and N = number of plate), were derived from the unbiased estimate of the second central moment and are thus independent of the particular distribution function with which the counts may conform

In order to determine the significance of differences between adjusted mean counts, standard errors,

$$s_{\bar{x}} = \frac{s}{\sqrt{N}},$$

were adjusted to conform with the adjustment of the means

$$\text{adjusted } s_{\bar{x}} = \frac{s_{\bar{x}}(\text{adjusted } \bar{x})}{\bar{x}}$$

This adjustment is dependent upon the assumption that a strict proportionality exists between the standard error and the mean, and may be only approximately correct   Consequently, most confidence may be placed in tests of significance of differences between means when the required adjustment is minimal

The relative error of the different plating methods was compared on the ba of the coefficient of variation,

$$V = \frac{s}{\bar{x}}$$

This definition of the coefficient of variation is that of Cramér (1946), and it differs from the usual percentage representation by a factor of 1/100   The standard deviation of the coefficient of variation,

$$s_r = \sqrt{\frac{s^2}{2N\bar{x}^2}\left(1 + 2\frac{s^2}{\bar{x}^2}\right)},$$

is also due to Cramér (1946)

Tests of significance of differences between the adjusted mean counts of d

were based on Student's ratio, s ot variation of different methods,

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(s_{\bar{x}_1})^2 + (s_{\bar{x}_2})^2}}$$

or

$$t = \frac{V_1 - V_2}{\sqrt{(s_{V_1})^2 + (s_{V_2})^2}}$$

Values of t exceeding the 5 per cent level of the t-distribution (1 960) for an infinite number of degrees of freedom are considered significant, those exceeding the 1 per cent level (2 576) highly significant

TABLE 1

Analysis of gravimetric measurements of water volumes delivered by three pipetting methods

STATISTICS	METHODS OF PIPETTING*		
	A	B	C
Number of replicates	60	60	59
Mean weights of water (grams)	0 1801	0 1025	0 9087
Calculated volumes of diluent (ml)†	0 1741	0 1025	0 9087
Standard deviations	0 0036	0 0028	0 0090
Standard errors	0 0005	0 0004	0 0012
Coefficients of variation	0 02001	0 02727	0 00989
Standard deviations of coefficients of variation	0 00183	0 00249	0 00090

* *Methods of pipetting*
A   Six drops delivered with capillary pipette
B   One tenth ml delivered with 1-ml serological pipette
C   Nine-tenths ml delivered with 1-ml serological pipette
† Calculations based on the ratio of drop volumes of distilled water and gelatin phosphate diluent

EXPERIMENTAL RESULTS

The results of gravimetric measurements of volumes delivered by the three pipetting methods are shown in table 1   The weights may be converted directly into volumes without appreciable error, since the density of water at 25 C is 0 9970 grams per ml (Hodgman and Holmes, 1940)   It may be noted that the capillary pipette used delivers in 6 drops 0 180 ml of distilled water or 0 174 ml of gelatin-phosphate diluent   The serological pipettes delivered slightly in excess of the volumes intended, and presumably the volumes were the same in the case of both water and diluent

The variability of even the most inaccurate method (B) was small, the coefficient of variation being less than 3 per cent of the mean   The coefficients of variation necessarily included errors of weighing, but these were apparently small   The variabilities of the three pipetting methods, however, differed appreciably   The order of increasing error was C, A, B in the ratio of 1 2 2 7   It

may be shown that the difference between any two of these coefficients of varia
tion was statistically significant and that method C was more accurate to a
highly significant degree than either of the other two   It may be concluded,
therefore, that of the methods tested most precise delivery was obtained when
0 9 ml were delivered from a 1-ml pipette, and the least precise when only 0 1 ml
was delivered from the same type of pipette   When it is necessary to use small
inocula of the order of 0 1 to 0 2 ml, the capillary pipette provides more accurate
delivery than do 1-ml pipettes

In order to compare the variabilities of the three pipetting methods with the
total error of the plate count, of which they are a part, two plating experiments
were conducted using the five plating methods described   The two experiments
were essentially repetitive   In experiment 1 plates were inoculated by methods

TABLE 2

Analysis of plate counts (experiment 1)

STATISTICS	PLATING METHODS*			
	A1	A2	B	C1
Number of replicates	66	64	67	49
Mean counts (colonies per plate)	293 14	308 86	178 34	153 37
Adjusted mean counts†	168 36	177 39	173 91	168 77
Standard deviations	21 949	22 928	13 999	11 727
Standard errors	2 7017	2 8660	1 7104	1 6753
Adjusted standard errors‡	1 5517	1 6460	1 6679	1 8135
Coefficients of variation	0 07488	0 07423	0 07850	0 07616
Standard deviations of coefficients of variation	0 00655	0 00660	0 00683	0 00777

* For plating methods, see text
† Values expected with 0 1 ml of 10⁻⁵ dilution
‡ Adjusted to conform with adjusted mean counts

A1, A2, and B from the 10⁻⁵ dilution, and plates and tubes by methods C1 and
C2, respectively, from the 10⁻⁶ dilution, of a single culture   In this experiment
the rolled tube counts were low and extremely erratic, presumably because of
having been held in the water bath at 45 C for 10 to 20 minutes after inoculation,
and therefore they were not included in the analysis   In experiment 2, which
employed a second culture, the dilutions used with each method were so chosen
that approximately equal mean counts could be expected with all method
These dilutions were 0 6 × 10⁻⁵ in the case of methods A1 and A2, 1 0 × 10⁻⁴
with method B, and 0 11 × 10⁻⁵ with methods C1 and C2   Furthermore, in
this experiment rolled tubes were rolled and cooled immediately after inocula
tion, a procedure requiring the attention of two operators

The analyses of these two experiments are shown in tables 2 and 3   Sin
rather similar values were obtained for the adjusted mean counts of the vari
methods, as well as for their variabilities as indicated by coefficients of varia
(which may legitimately be compared), it is necessary to resort to statistical te

ɔes between them   The significances
of the differences between all possible combinations of adjusted mean counts

TABLE 3

Analysis of plate counts (experiment 2)

STATISTICS	PLATING METHODS*				
	A1	A2	B	C1	C2
Number of replicates	56	56	56	56	56
Mean counts (colonies per plate)	152 91	161 36	158 30	159 05	167 48
Adjusted mean counts†	146 37	154 46	154 37	159 11	167 54
Standard deviations	12 807	13 568	11 513	14 157	12 338
Standard errors	1 7115	1 8132	1 5386	1 8919	1 6488
Adjusted standard errors‡	1 6382	1 7356	1 5003	1 8926	1 6494
Coefficients of variation	0 08376	0 08409	0 07273	0 08901	0 07367
Standard deviations of coefficients of variation	0 00797	0 00800	0 00691	0 00848	0 00700

* For plating methods, see text
† Values expected with 0 1 ml of 10^{-5} dilution
‡ Adjusted to conform with adjusted mean counts

TABLE 4

Significance of differences between mean counts of various plating methods

EXPERIMENT	METHODS* COMPARED	DIFFERENCES BETWEEN ADJUSTED MEANS	STANDARD ERRORS OF DIFFERENCES	STUDENT'S† RATIO
1	A1, A2	−9 03	2 262	3 992(++)
	A1, B	−5 56	2 278	2 440(+)
	A1, C1	−0 41	2 410	0 171
	A2, B	+3 47	2 343	1 482
	A2, C1	+8 62	2 471	3 487(++)
	B, C1	+5 14	2 486	2 070(+)
2	A1, A2	−8 09	2 387	3 389(++)
	A1, B	−8 00	2 221	3 602(++)
	A1, C1	−12 74	2 503	5 091(++)
	A1, C2	−21 18	2 325	9 110(++)
	A2, B	+0 09	2 294	0 038
	A2, C1	−4 66	2 568	1 813
	A2, C2	−13 09	2 394	5 467(++)
	B, C1	−4 74	2 415	1 964(+)
	B, C2	−13 18	2 230	5 910(++)
	C1, C2	−8 43	2 510	3 359(++)

* For plating methods, see text
† Ratios exceeding the 5% level of the *t*-distribution (1 960) are considered significant
(+), those exceeding the 1% level (2 576), highly significant (++)

within each of the two experiments are shown in table 4   Since these experiments
were not designed primarily for the comparison of mean counts, probably only a

appropriate pipetting methods as determined gravimetrically   The remaining variabilities (R) which complete the total are probably attributable to the distribution of organisms in the aliquots and to factors influencing the development of the organisms inoculated   Since the addition theorem is defined for terms of the same order as the variance (s^2), the relation between these components may be expressed by the equation,

$$T^2 = P^2 + R^2,$$

and R may be determined from the equation,

$$R = \sqrt{T^2 - P^2}$$

TABLE 5

Analysis of error involved in various plating methods

EXPERIMENT	PLATING METHOD*	COEFFICIENTS OF VARIATION			$T - R$	$\dfrac{100\,(T - R)}{T}$
		Total† (T)	Pipetting‡ (P)	Remainder§ (R)		
1	A1	0 07488	0 02001	0 07216	0 00272	3 63
	A2	0 07423	0 02001	0 07148	0 00275	3 70
	B	0 07850	0 02727	0 07361	0 00489	6 23
	C1	0 07646	0 00989	0 07515	0 00131	1 71
2	A1	0 08376	0 02001	0 08134	0 00242	2 89
	A2	0 08409	0 02001	0 08168	0 00241	2 87
	B	0 07273	0 02727	0 06742	0 00531	7 30
	C1	0 08901	0 00989	0 08846	0 00055	0 62
	C2	0 07367	0 00989	0 07300	0 00067	0 91

* For plating methods, see text
† Coefficients of variation taken from tables 2 and 3
‡ Coefficients of variation taken from appropriate entries in table 1
§ $R = \sqrt{T^2 - P^2}$ Additional preliminary analysis of this remainder variation (R), subtracting the variance due to a hypothetical Poisson distribution of organisms in aliquots of suspension, shows that residual variation is small and, in some cases, negative   There is at this time no plausible explanation for these negative residuals, but they suggest that there is something in the method which tends to keep the counts at an artificially even level, which would somewhat impair their use as measures of variation of bacterial populations

The values of these statistics are given in table 5 for all the plating methods of experiments 1 and 2   It will be seen that the remainder (R) constitutes almost the total error (T) The final column of this table lists the percentage contributions of the pipetting errors to the total errors when reduced to terms of the same order as the actual measurements   Taking both experiments into account, this amounted to only 2 9 to 3 7 per cent in the case of capillary pipetting methods, to only 6 2 to 7 3 per cent in the case of 0 1-ml aliquots delivered with a 1-ml pipette, and to only 0 6 to 1 7 per cent in the case of 0 9-ml aliquots This would appear to explain sufficiently the failure of the more precise methods of measurement to influence significantly the total error of the plate count

Experimental results have been presented which indicate that, within the limits of precision ordinarily employed in pipetting aliquots of inoculum into individual plates, the accuracy of measurement provides a negligible contribution to the total error, and that even so crude a method as that of measuring 0 1-ml quantities with a 1-ml pipette does not significantly increase the plating error Since a conclusion of this sort naturally leads to the recommendation of change in the technique of plate counting, it would be well to subject the experimental evidence to careful scrutiny

In the first place, the statistical analysis appears to be valid throughout E-sentially identical experiments, each employing numbers of replicates generally considered sufficient to determine the pertinent statistics, were mutually confirmatory   Estimates of error were independent of the form of distribution function which may be involved, and in at least one experiment the possible influence of unequal means was minimal   Tests of significance were necessarily based on a normal distribution function, but it is known that even with markedly skewed distributions the statistics concerned are approximately normally distributed

Also, it might be objected that the relative familiarity of the operators with the different techniques introduced a bias in favor of a particular method   It is especially probable that the coefficient of variation reported for the capillary dropping pipette method is greater than that which might be obtained by an experienced operator   However, this issue cannot be considered critical for the present study   Since the most inaccurate pipetting method did not appreciably affect the total plating error, further improvement of the relatively precise capillary-dropping method would, at most, have no greater effect

The specific coefficients of variation reported apply only under the experimental conditions described and with the particular organism used   The principal conclusion which is drawn from them, however, can be affected only by conditions or a bacterial species which leads to a *reduction* of the total error The extent to which the total error must be reduced in order that the pipetting error may exert an appreciable effect depends upon the magnitude of the pipetting error   The limiting value (T_p) of the total error corresponding to various percentage contributions (p) of the pipetting error (P) is found by solution of the equations,

$$p = \frac{100(T - R)}{T}$$

and

$$R = \sqrt{T^2 - P^2},$$

which is

$$T_p = \frac{P}{\sqrt{\dfrac{p}{100}\left(2 - \dfrac{p}{100}\right)}}$$

ting method used may be seen that an appreciable contribution (for example, 20 per cent of the total error) from the pipetting error is not attained until the total error is reduced to 0 016 to 0 045, depending on the pipetting method used It is questionable whether or not such low values for the total error are ever attained, in any real sense, with bacteriological plate counts Although coefficients of variation as low as 0 01 have been reported[2] for individual counts derived from small numbers of plates, such occurrences are related to the characteristic fluctuation of the statistic with small samples and cannot be regarded as accurate estimates Determinations based on large samples, or averages of numerous small samples, are rarely lower than our reported values

TABLE 6

Limiting values of total error (T_p)* *corresponding to various percentage contributions of pipetting error*

PIPETTING ERROR PERCENTAGE OF TOTAL (p)†	METHODS OF PIPETTING‡		
	A	B	C
1	0 1419	0 1934	0 0701
5	0 0641	0 0874	0 0317
10	0 0459	0 0625	0 0227
20	0 0334	0 0454	0 0165
33	0 0270	0 0368	0 0133
50	0 0231	0 0315	0 0114

$$* T_p = \frac{P}{\sqrt{\frac{p}{100}\left(2 - \frac{p}{100}\right)}}$$

$$† p = \frac{100(T - R)}{T}$$

‡ Methods of pipetting
A Six drops delivered with capillary pipette $(P = 0\ 02001)$
B One-tenth ml delivered with a 1-ml serological pipette $(P = 0\ 02727)$
C Nine-tenths ml delivered with a 1-ml serological pipette $(P = 0\ 00989)$

Since, then, there is every reason to believe that the conclusion derived from the experimental results is valid for general application, it is clear that efforts to improve the accuracy of bacteriological plate counting methods should at present be directed elsewhere than toward increased precision of measuring aliquots into plates Two plausible sources of the remaining variability were indicated in the introduction One of these, the distribution of organisms in the aliquots, has received considerable attention (Fisher, Thornton, and Mackenzie, 1922, James and Sutherland, 1939, Sutherland and James, 1938, Wilson and Kullmann, 1931) and is presumably susceptible to precise mathematical definition If this is the case, the contribution of this particular source of variability may be reduced

[2] This figure is calculated from a value given by Jennison (1937) for the standard deviation of the mean (standard error) of 5 plates, expressed as percentage of the mean

to whatever extent desired by sufficiently increasing the number of plates inocu lated It may be assumed that the standard error of replicate plates is approxi mately inversely proportional to the square root of the number of plates from which it is determined Therefore, whatever the error obtained with the con ventional usage of 3 plates, for example, it may be halved by the use of 12 plate or reduced by $\frac{2}{3}$ or $\frac{3}{4}$ by the use of 27 or 48 plates, respectively Investigators are discouraged from making full use of this relationship by the inconvenience and expense involved in the preparation, inoculation, and counting of large num bers of plates The present study provides some encouragement in this direc tion, in that it justifies the use of a plating method (B) with which large numbers of plates (9 to 18) may be as rapidly and conveniently inoculated as could small numbers (3 to 5) with standard methods In addition, preparation of the plate is facilitated because they may be prepared on a large scale at convenient inter vals and stored

The other source of variability indicated in the introduction is more difficult to assess, but it has been shown (Harmsen and Verweel, 1936–1937) that in cer tain cases, at least, conditions that might be expected to favor the development of the inoculated bacteria also tend to bring the plate counts into better agree ment with the Poisson distribution function, hence this type of study might be used to assess the suitability of various media It would appear that this pos 1 bility merits more extensive investigation

In view of all these considerations it is possible to make recommendations with respect to that part of the dilution plate count procedure subsequent to the prepa ration of dilutions When the growth requirements of the bacterial species per mit, prepared and dried plates should be inoculated with 0 1-ml aliquots delivered by means of 1-ml serological pipettes, and the inoculum should be distributed over the surface with a sterile glass rod This method is quite as precise as any of those studied and has the advantage that one source of variability may con veniently be decreased by approximately 42 or 59 per cent by increasing the number of plates inoculated from 3 to 9 or 18 Using method B of the present study for an example, a standard error of 4 37 per cent of the mean count is pro vided by 3 plates, is reduced to 2 52 per cent with 9 plates, and to 1 78 per cent with 18 plates These figures, like all the data presented, are exclusive of any error contributed by the process of preparing dilutions In the case of an organ ism apparently characterized by a larger variability, as for example *Bacterium tularense* (Snyder, Engley, Penfield, and Creasy, 1946), the reduction of the standard error would be more impressive

It is recognized that frequently practical limitations with respect to the numbe of plates inoculated are set by considerations of economy instead of manipulative convenience, so that the foregoing recommendations may well be referred princi pally to studies in which precision is of especial importance, rather than to rou tine practice Even in the latter case, however, use of the surface plating technique with the usual number of 3 to 5 plates may be recommended on the basis of saving time and effort

The rolled tube method may be recommended in cases where strict economic

⎯ ⎯ ' ⎯ ⎯ ' ⎯ is suggestive evidence
that this method will give a higher, and therefore a presumably more accurate,
count   In our opinion, however, this method is not sufficiently convenient for
large-scale studies

ACKNOWLEDGMENT

SUMMARY

Five bacteriological plate counting methods were compared with respect to
the magnitude and reliability of the counts obtained with *Shigella dysenteriae*
The coefficients of variation of the five plating methods were not significantly
different, even though the coefficients of variation of the three methods of meas-
uring aliquots of inoculum into plates did differ significantly   This phenomenon
was referred to the relative magnitudes of the plating errors (coefficients of varia-
tion $= 0\ 0727$ to $0\ 0890$) and of the pipetting errors (coefficients of variation $=
0\ 0099$ to $0\ 0273$)   It was demonstrated that no significant contribution to the
total error can be expected from the pipetting methods used unless the total
error is considerably less than that observed in this study

Efficient spreading of the inoculum in the case of surface plating methods
significantly increased the counts obtained   The rolled tube method gave a
significantly higher count than the poured plate method

As a result of these findings it is possible to recommend more extensive use of
surface plate counting methods, which are conducive to greater replication of
plates

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The primary stimulus to the following experiments was a need for a method whereby large numbers of the yeastlike cells of *H capsulatum* could be easily obtained   A fluid medium in which the yeastlike phase would multiply rapidly and extensively and not revert to the mycelial form would present distinct advantages over blood agar slants   Accordingly, investigations were initiated to determine more fully the cultural requirements and characteristics of the yeast like phase, and thereby to develop a liquid medium for its maximum growth

MATERIALS AND METHODS

The 19 strains of *H capsulatum* used were all obtained from the collection maintained in this laboratory   Saline suspensions of 3-to-4-month-old Sabouraud's agar cultures of mycelium and conidia were injected intraperitoneally into mice   After 2 to 5 weeks the mice were killed and the yeastlike phase of all but two of the strains was recovered on 20 per cent rabbit blood agar slants from the liver, spleen, or kidney   This phase was maintained at 37 C on 20 per cent rabbit blood agar slants in tubes which had been sealed to prevent drying of the agar   The cultures were transferred once a week, although every 2 to 3 weeks proved satisfactory for the maintenance of the yeastlike phase   In the following investigations, the fungus was grown at 37 C in 10 ml of the semisolid medium (to be described) in a 25-by-150-mm tube, which in most cases was sealed with paraffin to prevent evaporation

EXPERIMENTS AND RESULTS

Since the yeastlike phase was known to exist, and possibly grow, in the blood of man and animals, and since blood and blood serum had been shown by De Monbreun to maintain the yeastlike phase, a strain of *H capsulatum* (6521) was inoculated into several liquid media in which sheep serum or plasma was the basic substance   Although cultures in each of these media were incubated at 37 C for 3 weeks under different oxygen tensions, no marked growth of the yeastlike phase was apparent

Many other variations of the medium were tried, but in all there appeared very little, if any, growth of the yeastlike phase   However, when the viscosity of the medium was increased by the addition of 0 175 per cent Difco agar, the yeastlike phase grew most abundantly and was nearly free of mycelium   The term "nearly free" is used since examination of several slides containing the yeastlike phase would reveal only an occasional abortive hypha   The medium on which this profuse growth appeared had the following composition (Difco products) proteose peptone, 10 g, neopeptone, 3 25 g, tryptone, 3 25 g, glucose, 2 0 g, sodium chloride, 5 0 g, disodium phosphate, 2 5 g, agar, 1 75 g, and distilled water to make 1,000 ml   This medium was the basic one used in most of the ensuing experiments and will be referred to hereafter as "YP medium "

Hydrogen ion concentration   The YP medium was dispensed in 10 ml amounts in 25-by-150-mm culture tubes, and duplicate tubes were adjusted to pH readings of 3 9, 4 3, 4 85, 5 4, 5 85, 6 3, 6 9, 7 05, 7 3, 7 5, 7 7, 8 1, 8 6, and 9 6   The tubes

The results showed maximum growth of the yeastlike phase at pH's between
6 3 and 8 1   In these tubes, as well as those in which less growth occurred, al-
most no mycelium was found   The experiment was repeated with two other
strains, and similar results were obtained

Temperature   In order to determine the optimum temperature for growth of
the yeastlike phase, 7 strains of *H capsulatum* were grown at 4 temperatures, 25,
31, 37, and 43 C, in YP medium   The inoculum in all cases was the yeastlike
phase

At 25 C and at 31 C the resultant growth was mycelial in character, whereas at
37 C the yeastlike phase grew extensively with no mycelium evident   At 43 C,
with the exception of one strain which showed extensive growth, the yeastlike
phase grew much less abundantly than at 37 C, although again no hyphae ap-
peared   Accordingly, of the temperatures studied the best growth of the yeast-
like phase occurred at 37 C
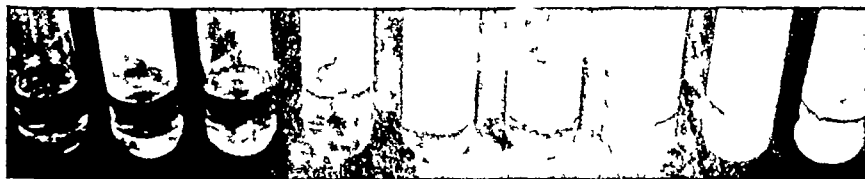
FIG 1   GROWTH OF THE YEASTLIKE PHASE OF STRAIN 6521 OF H CAPSULATUM AT pH 7 3 IN
YP MEDIUM IN AGAR PERCENTAGES OF 0 0, 0 005, 0 01, 0 05, 0 1, 0 2, 0 3, 0 4, AND
0 5, (FROM LEFT TO RIGHT)

Viscosity   In tubes containing 10 ml of the basic YP medium, the percentage
of agar was varied from 0 0 to 0 5 as follows   0 0, 0 005, 0 01, 0 05, 0 1, 0 2, 0 3,
0 4, and 0 5 per cent   Each of these percentages was inoculated in duplicate
with the yeastlike phase of strain 6521 and incubated at 37 C

After 3 weeks' incubation examination of the cultures revealed marked develop-
ment of the yeastlike phase in the tubes containing an agar percentage of 0 1 and
higher   Virtually no growth was discernible in the tubes with agar percentages
of 0 05 and less   The most cells developed in the medium containing 0 2 per cent
agar, with slightly less in the one having 0 1 per cent agar   The yeastlike colo-
nies in the YP media with agar percentages of 0 3 to 0 5 were extensive and also
unmixed with mycelial elements, but, owing probably to the solidity of the
medium, they were restricted to the very surface of the medium (figure 1)   Ad-
ditional studies at agar concentrations between 0 1 and 0 3 per cent indicated that
0 175 per cent agar provided a viscosity for the most extensive development of
the yeastlike phase, to the exclusion of the mycelial phase

These experiments to determine the optimum viscosity of the medium for the
growth of the yeastlike phase of *H capsulatum* were repeated with 5 other strains,
and results similar to those obtained with strain 6521 were obtained   When

growth was extensive, it appeared in the upper portion of the medium (figure 1) When growth was markedly restricted, as in the tubes with no agar, no yeast cell were apparent in the upper part of the medium, although very small quanti ... developed at the bottoms of the tubes

The reason for the luxuriant growth of the yeastlike phase in a medium with a low percentage of agar was obscure Two possible explanations were tested (1) the agar contained an essential growth substance, or (2) the agar produced a narrow zone of a particular oxygen or carbon dioxide tension essential for the development of the yeastlike phase Experiments were conducted to test these two hypotheses

Difco agar, which was the type used in the foregoing experiments, was washed for 48 hours in slowly running tap water and then incorporated into tubes of YP medium in a percentage of 0 175 These tubes and similar ones without aga were inoculated in duplicate with 12 strains of *H capsulatum* and incubated at 37 C for 3 weeks Again, in all cases, growth of the yeastlike phase was luxuriant in the tubes of 0 175 per cent agar and virtually nonexistent in those containing no agar

In order to test the possibility that some inorganic element was exerting an effect on the growth of the yeastlike phase, as much as ten times the concentration of agar used in the YP medium, namely up to 1 75 g per 100 ml of medium was ashed and added to the medium The tubes were then inoculated with 7 strains and incubated at 37 C for 3 weeks In no case did the yeastlike phase appear

To eliminate competely the possibility of there being some necessary organic or inorganic growth substance in the agar, semisolid "silica gel" was prepared according to the method of Anderson and MacSween (1942), with the exception that a ratio of one part silicate solution to 25 parts of nutrient medium was employed instead of the 1 9 ratio recommended The purpose of the latter modification was to produce a medium of approximately the same viscosity as containing 0 175 per cent agar The nutrient base was YP medium in all cases The silica gel semisolid medium, after inoculation with 14 strains and incubated at 37 C for 3 weeks, brought about luxuriant growth of the yeastlike phase in all cases but one, with virtually no mycelial fragments discernible

Oxygen requirements Since the yeastlike phase grew near the surface of YP medium, the possibility presented itself that the organism concerned was strongly aerobic and that the semisolid YP medium served to keep the cell at or near the surface of the medium Accordingly, experiments were initiated determine the oxygen requirements of the cells

Tubes containing 10 ml of the YP medium were inoculated with the yeastlike phase and incubated at 37 C under each of the following conditions (1) in complete anaerobiosis (produced by a suction pressure of 30 inches of mercury) under one-half inch of sterile liquid petrolatum (3) under 10 per cent carbon dioxide in air (4) under 20 per cent carbon dioxide in air, (5) under 40 per cent carbon dioxide in air (6) under 80 per cent carbon dioxide in air, and (7) 100 per cent oxygen

' y ' ₍ѕ e ₎ ' to »‹ ' y room air at 37 C   Under sterile liquid petrolatum the same 5 strains multiplied again only in the yeastlike phase, although to a slightly lesser degree than the controls   Under 10, 20, 40, and 80 per cent carbon dioxide, luxurious development of the yeastlike phase was observed in all cases, with virtually no abortive hyphae present   When in an atmosphere of 100 per cent oxygen, growth of the yeastlike phase was somewhat less than the controls, with one strain having a noticeable amount of mycelial development

In addition, a flask with YP medium minus agar was inoculated with the yeastlike phase and shaken constantly and vigorously for 4 weeks at 37 C   No extensive growth of the yeastlike phase was then apparent   However, if the inoculum of yeastlike cells was carefully floated on cork shavings, oil drops, or paraffin chips on the surface of YP medium minus agar, marked growth of the yeastlike phase resulted on the surface of the medium

Age of culture   The yeastlike phase of *H capsulatum* was maintained in culture both on 20 per cent rabbit blood agar slants and in YP medium   The cultures on the blood agar were transferred every 7 days, whereas those in the YP medium were transferred only once a month   Nevertheless, several times isolates on the blood agar slants spontaneously reverted to the mycelial phase and had to be restored by inoculations from the YP medium   This spontaneous transformation to mycelium at no time occurred in the YP medium   Thus, it would seem that the semisolid medium is more suited for the maintenance of the yeastlike phase in culture

Growth of other species on YP medium   Since the yeastlike phase of *H capsulatum* grew so well on YP medium, it seemed logical to inoculate other species of pathogenic fungi therein to obtain their respective parasitic phases   The species studied in this regard were *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Sporotrichum schenckii*

When the yeastlike phase of *B dermatitidis* from rabbit blood agar slants was inoculated into the YP medium and incubated at 37 C, the 5 strains tested developed primarily as mycelium   In the studies on 6 strains of *C immitis*, with inocula consisting of either a saline suspension of conidia or a suspension of spherules from an infected mouse testis, there was no obvious development of the yeastlike phase in the YP medium at 37 C   Of the 6 strains of *S schenckii* incubated in the YP medium, two developed entirely in the yeastlike form, but the others appeared both in the mycelial and budding phases

SUMMARY

Seventeen strains of *Histoplasma capsulatum* were grown in a fluid medium in the yeast-like phase

The best growth occurred at hydrogen ion concentrations between 6 3 and 8 1, at a temperature near 37 C, and in a medium containing a mixture of organic nitrogen compounds

growth was extensive, it appeared in the upper portion of the medium (figure 1) When growth was markedly restricted, as in the tubes with no agar, no yeast cell were apparent in the upper part of the medium, although very small quantities developed at the bottoms of the tubes

The reason for the luxuriant growth of the yeastlike phase in a medium with a low percentage of agar was obscure   Two possible explanations were tested (1) the agar contained an essential growth substance, or (2) the agar produced a narrow zone of a particular oxygen or carbon dioxide tension essential for the development of the yeastlike phase   Experiments were conducted to test these two hypotheses

Difco agar, which was the type used in the foregoing experiments, was washed for 48 hours in slowly running tap water and then incorporated into tubes of YP medium in a percentage of 0 175   These tubes and similar ones without agar were inoculated in duplicate with 12 strains of *H capsulatum* and incubated at 37 C for 3 weeks   Again, in all cases, growth of the yeastlike phase was luxuriant in the tubes of 0 175 per cent agar and virtually nonexistent in those containing no agar

In order to test the possibility that some inorganic element was exerting an effect on the growth of the yeastlike phase, as much as ten times the concentration of agar used in the YP medium, namely up to 1 75 g per 100 ml of medium, was ashed and added to the medium   The tubes were then inoculated with 7 strains and incubated at 37 C for 3 weeks   In no case did the yeastlike phase appear

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Oxygen requirements   Since the yeastlike phase grew near the surface of the YP medium, the possibility presented itself that the organism concerned is strongly aerobic and that the semisolid YP medium served to keep the cell on or near the surface of the medium   Accordingly, experiments were initiated to determine the oxygen requirements of the cells

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A NOTE ON FORMATE RICINOLEATE LACTOSE BROTH

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In the formate ricinoleate medium recommended in *Standard Methods of Water Analysis*, gas production may be due to the decomposition of either lactose or the salt of formic acid in the medium   In case the latter is the source of gas, the reaction of the medium turns alkaline, whereas if the former (lactose) is decomposed an acid reaction will result   In the standard methods formula no indicator to determine the reaction of the medium is included, with the result that considerable additional work is necessary in order to ascertain whether the gas former is probably a lactose fermenter

In the course of collateral studies, it was noted that all cultures of *Escherichia*, *Citrobacter*, and *Aerobacter* produced acid and gas, whereas those of the genera *Proteus*, *Salmonella*, and the paracolon bacilli produced gas with an alkaline reaction when formate ricinoleate broth was employed

In *Standard Methods for Water Analysis*, 9th edition, it is pointed out, in conjunction with the completed test, that if gas is formed in formate ricinoleate broth, inoculated from an agar slant showing sporeformers and gram-negative rods, the probable presence of the coliform group of organisms should be verified by inoculation from the formate ricinoleate to a tube of standard lactose broth and to a new agar slant   The objective here is to determine whether the gas produced was due to lactose fermentation   If, however, an indicator were present in the formate ricinoleate broth, this last step could be dispensed with as far as detection of a lactose-fermenting organism is concerned

Standard Methods, furthermore, states that, if in this last step spores are present on the agar slant, then for all practical purposes organisms of the coliform group may be considered absent   In view of the fact that gram-negative rods were originally present on the agar slant from which the formate ricinoleate broth tube was inoculated and that the paracolon bacilli, *Proteus*, and *Salmonella* produce gas with an alkaline reaction in formate ricinoleate broth, the conclusion that typical coliforms are absent when spores are found on the agar slant, though correct, may (if gram-negative rods are still present with the spores) actually result in missing the presence of *Salmonella* and the slow lactose-fermenting paracolon bacilli, which might actually be of sanitary significance

It is suggested, therefore, that the simple addition of an acid-base indicator in the formate ricinoleate medium might serve to eliminate further work when both acid and gas are produced, and the objective is merely to detect typical coliform bacteria, whereas the production of an alkaline reaction in conjunction with gas would serve to facilitate detection of *Salmonella* and paracolon bacilli, if present

EOSIN METHYL-GREEN SULFITE AGAR A MODIFICATIO\ OF LEVINE'S E M B AGAR

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Most of the differential media for enteric bacteria are based on the fermention of lactose   The splitting of this carbohydrate gives rise to acid as well a oxidizing products which change the color of the indicators employed   As th lactose-containing media give a definite advantage for the growth of the lactose fermentng coli-aerogenes group, many of the media are supplemented with selative inhibitors (appropriate concentrations of bile salts, brilliant green, etc) As a result of this, one has to employ different media for best results in the isola tion of either *Escherichia*, *Salmonella*, or *Shigella*   There is, therefore, a need for a medium on which *Escherichia*, *Salmonella*, and *Shigella* would grow well, but on which *Escherichia* would not profit too much by its lactose fermenting ability

Levine's E M B agar is probably the best medium for the isolation and charac terization of the coli-aerogenes group, but the high toxicity of the methylene blue prevents the growth of some members of the genera *Salmonella* and *Shigella* Taking advantage of the fact that aniline dyes are much less toxic in their re duced (colorless) form (Dubos J Exptl Med , 49, 575), methyl green reduced by sodium sulfite was employed to replace the methylene blue of Levine's E M.B The low toxicity of the reduced methyl green permits a good growth of the la tose-negative enteric strains   Furthermore, the advantage experienced by th lactose-fermenting strains on this lactose-containing medium is counterbalanced by the oxidation of the methyl green and its subsequent increase in toxicity against the lactose fermenters

The eosin methyl-green sulfite (E M G S) agar is prepared in the following manner

A To distilled water   1,000 ml
   Add proteose peptone   10 g
        lactose (Difco)   2o g
        1% solution of methyl green   15 ml
B Decolorize (until only faint color remains) with a 10% solution of sodium sulfite, adding a drop at a time (will require 1 5 to 2 0 ml per liter of media)
C Add 2% solution of eosin Y   7 5 ml
        agar   15 g
D Boil to dissolve completely and sterilize in the autoclave at 16 pounds' pressure (121 C) for 15 minutes

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ι   ιv   that some samples of agar interfere with the proper reduction of the methyl green)

This medium, at the same time, indicates pH and rH changes without sodium sulfite, it is equivalent to Levine's E M.B , and without eosin it is equivalent to Endo's medium   It gives, therefore, a wide range of different shades among lactose-fermenting as well as lactose-nonfermenting colonies   Changes in color, resulting from the fermentation of lactose, do not diffuse around the colony as in Endo's medium but are localized to the center of the fermenting colony as in Levine's E M B   The appearance of the coli-aerogenes strains on this medium is similar to that on E M B , and lactose-negative strains form larger colonies with shades varying from gray to red according to the alkalinity produced *Shigella dysenteriae* strains, which failed to grow on Levine's E.M.B , gave good-sized colonies on this medium   Except for enterococci, which produce very small colonies, E M G S agar completely inhibits gram-positive organisms

ANAEROBIC OXIDATION OF HYDROCARBONS BY SULFATE-REDUCING BACTERIA[1]

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Assimilation of aliphatic hydrocarbons by anaerobic bacteria has been noted in the case of *Desulfovibrio* species by Novelli and ZoBell (J Bact, 47, 447) The following data reveal that these sulfate reducers catalyze the anaerobic oxidation of a considerable variety of mixed hydrocarbons Substrates were emulsified in water with gum arabic, autoclaved, mixed with sterile mineral salts solution, and inoculated with mixed cultures of sulfate-reducing anaerobes Cultures were incubated in 60-ml glass-stoppered bottles at 27 C Hydrocarbon oxidation was accompanied by sulfate reduction, which was employed as an index of bacterial activity Sulfates were not reduced in the presence of gum arabic mixtures containing no hydrocarbons

SUBSTRATE	NUMBER OF CULTURES TESTED	NUMBER THAT WERE ACTIVE
Crude oils		
Calif crude no 138-4	27	27
Calif crude no 143-3	27	27
Calif crude no 144-1	27	26
Pa crude no 148-2	29	28
Pa crude no 148-4	29	29
Refinery products		
Kerosine no 109-5	29	28
Lubricating oil no 140-2	27	26
Paraffin oil	27	26
Gum arabic (control)	29	0

Long-chain aliphatic hydrocarbons have undergone rapid destruction by sulfate reducers Insoluble fatty acids were isolated as intermediates in the consumption The presence of fatty acids, however, was transitory, for they underwent further degradation The utilization of hexadecane was vigorous and was traced quantitatively by the ether extraction procedure of Go and (Science, 98, 546) and the fractionation methods of Wilson and Hansen (J B Chem, 112, 457) A typical experiment, employing culture XXIX 130-1, recorded below

DAYS OF INCUBATION	EXTRACT FRACTIONS	
	Unsaponifiable (mg/L)	Fatty acid (mg/L)
0	910	0
13	568	120
30	48	0

Hydrocarbon utilization by *Desulfovibrio* species appears to be associated with the presence of a dehydrogenase system   This enzymatic activity has been detected by employing washed cells of pure sulfate-reducing species in the Thunberg method   Substrates such as hexadecane or hexadecene are well suited to the procedure and, upon activation, readily donate hydrogen to methylene blue Nonsulfate-reducing anaerobes known to be incapable of the cultural utilization of hydrocarbons were included as controls   Tubes prepared without hydrocarbon demonstrated no dehydrogenation of the gum arabic normally used to emulsify the substrates

SUBSTRATE	SULFATE REDUCERS		NONSULFATE REDUCERS	
	Tested	Active	Tested	Active
Hexadecane	3	2	4	0
Hexadecene-1	3	3	4	0
Gum arabic (control)	3	0	4	0

In Vivo Antibacterial Activity of Hexanolactone. *Charles B. Johnson* and *J. B. Bartholomew*, Department of Bacteriology, University of Southern California

The antibacterial agent hexenolactone occurs naturally in whole oranges, baked whole meal bread, and ungerminated cereal. This compound was recently synthesized by Meddwar, Robinson and Robinson (1943) and Kuhn and Jerchel (1948). Bartholomew and Hervey (1947) demonstrated the *in vitro* inhibitory effect of this compound at varying concentrations against a variety of pathogenic and nonpathogenic gram positive and gram negative bacteria. Particular effectiveness was noted in the case of *Salmonella enteritidis*.

The first experiment in this *in vivo* study was designed to determine the LD$_{50}$ of hexenolactone for 14- to 18-gram white mice. The chemical was diluted in physiological saline and administered by the intraperitoneal route. Death was used as an end point of toxicity. All observations were made at the end of a 96 hour period. The LD$_{50}$ of hexenolactone calculated according to the method of Reed and Muench (1937) was 5.26 mg per 14- to 18-gram mouse. A 1 mg dose of hexenolactone was selected as the therapeutic dose.

Experiments using varying dosages of *Salmonella enteritidis* and a single 1 mg injection of hexenolactone administered 30 minutes after injection of organisms by the intraperitoneal route showed protection ranging from 0 per cent, at the highest dosage of organisms, to 100 per cent, at the lowest dosage of organisms. Using a constant dosage of approximately 100,000 organisms, protection ranged from 50 to 60 per cent for the treated mice.

An unsuccessful attempt was made to increase the rate of survival, using *Salmonella enteritidis*, by administering 1 mg of hexenolactone 30 minutes after injection of the organisms and 1 mg 8 hours after the initial injection of the chemical. Preliminary observations indicate a possible decrease in the rate of survival with increased amounts of the chemical.

Report of a Relatively Severe and Prolonged Diarrhea Presumably Due to Salmonella pullorum from the Ingestion of Incompletely Cooked Eggs. *T. F. Judefind*, Department of Pathology and Bacteriology, College of Medical Evangelists, Loma Linda, California

Although *Salmonella pullorum* had formerly been considered nonpathogenic for man, a number of cases of human diarrhea from which this organism has been isolated have been reported. Attention has recently been drawn to this subject by the report of Mitchell *et al.* (J. Infectious Diseases 79, 57–62, 1946), which gives data on a major food-poisoning outbreak characterized by a diarrhea averaging 2 to 8 days and involving 128 persons, 172 of whom required hospitalization. *Salmonella pullorum* was rather definitely incriminated in this outbreak and the available evidence indicated that the source of the organism was incompletely cooked eggs in rice pudding.

A case is reported of a female, age 29, who developed a diarrhea 8 days after hospital entry for obstetrical care. This diarrhea lasted for about a month. Just before recovery there was an acute exacerbation associated with a temperature of 102.6 F that required a second hospitalization. *Salmonella pullorum* was isolated from the patient's stool at the onset of the diarrhea. The source of the infection appeared to be incompletely cooked eggs which were served for breakfast each morning while the patient was hospitalized for obstetrical care.

THE EFFECT OF STREPTOMYCIN ON THE METABOLISM OF BENZOIC ACID BY CERTAIN MYCOBACTERIA[1]

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Benzoic acid and some related compounds are able to increase the metabolism of mycobacteria (Bernheim, 1941) The oxygen uptake and carbon dioxide production of the pathogenic strains H-37 and B_1 are greatly accelerated when small amounts of benzoic or salicylic (o-hydroxybenzoic) acid are added to the bacterial suspensions Other hydroxybenzoic acids as well as aminobenzoic acids are without effect Benzoic and salicylic acid are not oxidized by these pathogenic strains, so presumably they act to catalyze some endogenous metabolic reaction of the cell A *Mycobacterium* obtained from the collection of Dr Van Niel, recently identified as *M lacticola*, oxidizes benzoic, m- and p-hydroxybenzoic acids but not salicylic acid (Bernheim, 1942) The first three acids can act as the sole carbon sources for the growth of this species (Saz and Bernheim, 1942) The metabolism of a number of other mycobacteria with respect to these compounds may be said to be intermediate between that of *M lacticola* and the pathogenic strains in the sense that benzoic acid is oxidized by them but the hydroxy acids are not, although some of the latter may increase the oxygen uptake Lehmann (1946) has found essentially the same effects in the mycobacteria he has studied Because of the apparent importance of benzoic acid in the metabolism of these organisms, it was of interest to study the effects of streptomycin on its oxidation

EXPERIMENTAL

Cultures The following nonpathogenic strains of *Mycobacterium* obtainable from the American Type Culture Collection were used *M stercoris* (77), *M avium* (9077), *M tuberculosis* var *bovis* (BCG 8240), *M tuberculosis* var *bovis* (599), *M leprae* (4244), and *M tuberculosis* (607) All of these strains attain full growth in 3 days at 37 C on Long's synthetic medium and thus grow more rapidly than their freshly isolated virulent counterparts

The H37 and B_1 strains of *M tuberculosis* were originally obtained from Saranac Lake and have been maintained in these laboratories for several years

Media *M lacticola* and *M stercoris* were grown at room temperature on the medium described by Kohn and Harris (1941) in which glucose is the sole carbon source For special tests the glucose was replaced by 0 1 per cent benzoic acid or m-hydroxybenzoic acid

The pathogenic H37 and B_1 strains were grown on veal infusion glycerol bi whereas the other mycobacteria were grown on Long's synthetic medium 37 C   For special tests the following modifications of the medium de inb by Dubos *et al* (1946) were used

Asparagine (or benzoic acid)	1 0 g
Ammonium citrate	1 0 g
KH_2PO_4 12H$_2$O	1 0 g
Na HPO$_4$ 12H O	6 3 g
Ferric ammonium citrate	0 1 g
$MgSO_4$ 7H$_2$O	0 6 g
H$_2$O	to 1,000 0 1 l

The pH was adjusted to 7 0   This medium was also used with the addition 2 0 per cent agar

Cultures were used when they were at the beginning or middle of the lo rithmic growth phase   Even suspensions of washed bacteria were made by t method already described (Bernheim, 1941)   An aliquot (3 to 7 mg dry we of bacteria) in 2 0 ml of м 20 phosphate buffer pH 6 7 was placed in each W ir vessel and the oxygen uptake measured with and without the addition of v ir compounds   Streptomycin HCl (Merck) was used   It was made up in и and diluted with the phosphate buffer immediately before its addition to tl suspension   All experiments were done at 37 C in air

The first experiments showed that the oxidation of benzoic acid by m t these bacteria is markedly sensitive to streptomycin   As shown in figure 1 l' μg in 2 0 ml causes an appreciable inhibition, and with 10 0 μg in 2 0 ml tl inhibition is almost complete   Figure 1 also shows that 500 μg of strept in SO$_4$ or streptidine HCl (kindly supplied by Merck and Company) are with effect   Apparently the whole streptomycin molecule is necessary for the ir! tion of the oxidation of benzoic acid, as it is for the inhibition of growth   s n tomycin, however, has no effect on the metabolism of the pathogenic h ct which do not oxidize benzoic acid   Even 500 μg have no effect on the incre uptake in the presence of benzoic and salicylic acids and have little effect on t oxidation of fatty acids and other substrates   Pathogenicity in these r bacteria is accompanied by a change in metabolism and a greatly diff sensitivity of oxidative reactions to streptomycin   This difference is not rel in the effects of streptomycin on the growth of these organisms

It was then necessary to determine whether the oxidation of benzoic   l the reaction most sensitive to streptomycin   Various possible substr added to suspensions of the bacteria   No amino acid tested is oxidized   t exception of tyrosine by *M lacticola* and asparagine by some of the other Higher and lower fatty acids are oxidized rapidly by all of them   In l the only sugar which is readily oxidized by most of the strains, the being the pathogenic H37 and *M stercoris*   Trehalose is a disaccharide cosido-1-α-glucoside) containing two molecules of glucose linked thr aldehyde groups   Other glucose disaccharides, such as cellobio   ardi

than any of the other species   Fructose is oxidized slowly by most of the organisms, as are mucic acid, mannitol, and sorbitol   These results are shown in table 1, which also indicates which compounds can act as sole sources of carbon for growth   Figures 2 and 3 show the effects of streptomycin on the oxidation of a number of these compounds in comparison with its effects on the oxidation of
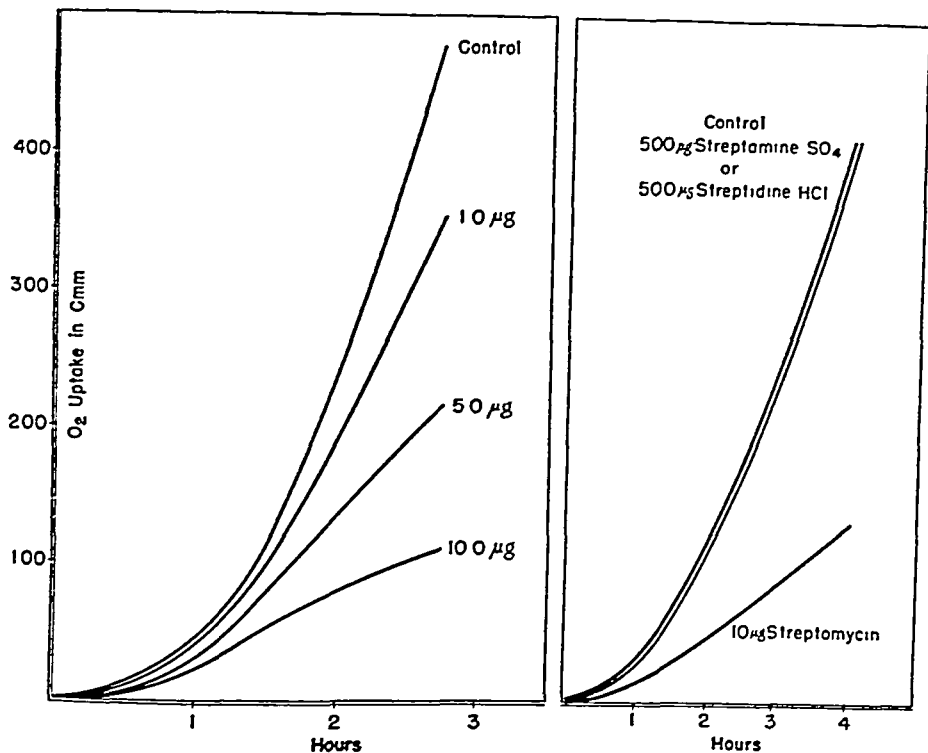
FIG 1 *Left* Effect of different amounts of streptomycin on the oxidation of benzoic acid by *M* 607   *Right* The effect of streptomycin, streptamine, and streptidine on the oxidation of benzoic acid by *M* BCG

benzoic acid   In all cases the oxidation of benzoic acid is inhibited to a greater extent than that of any other compound tested   *M lacticola* oxidized *m*- and *p*-hydroxybenzoic acids as well as phenol and tyrosine   The oxidation of these hydroxy compounds is somewhat more sensitive to streptomycin than that of benzoic acid, but all these reactions require 50 μg per ml of streptomycin for inhibitions comparable to those obtained by 5 0 μg per ml in all the other non-pathogenic species

The next question which arose was whether organisms made resistant to streptomycin by growing them in gradually increasing concentrations of the drug would show a corresponding resistance to the effect of this drug on the oxidation

of benzoic acid These experiments were done on *M lacticola* and *Mycob.*
rium 607 The results are shown in table 2 and indicate that a parallelism ex-
between the ability of these bacteria to grow in streptomycin and their ability to
oxidize benzoic acid in its presence Since streptomycin-resistant organisms d ?
not inactivate the drug, it is possible to assume provisionally that resistance to ?
is accompanied by the production of more of the catalyst or catalysts respon .?
for the oxidation of benzoic acid Support is given to this assumption by th?
following experiments *M lacticola* was grown in a medium with benzoic ???
as the sole carbon source The oxidation of benzoic acid by these organisms w?
not inhibited by 300 μg per ml streptomycin, whereas the oxidation by the control
grown in glucose was inhibited as usual by 50 μg per ml The oxidation of
p-hydroxybenzoic acid by the benzoate strain was as sensitive to streptomycin
as the oxidation of this compound by the control This proves, incidentally,
that the oxidation of benzoic acid by *M lacticola* does not go through the h?

TABLE 1

Properties of the cultures

ORGANISM	BENZOIC ACID	SALICYLIC ACID	*p*-HY-DROXY BENZOIC ACID	TREHA LOSE	FRUCTOSE	GLUCOSE	MUCIC ACID	ACETIC ACID	PALMITI ACID	X??? ???
M leprae	+ C	−	−	+		−		+	+
M 607	+ C	− X	− X	+ C	+ C	+ C	+ X	+	+	+ C
M stercoris	+ C	−	−	−	+	− C		+	+
M avium	+ X	−	−	+		−			
M BCG	+ C	−	−	+	+	− C	+	+	+	+
M phlei	−	−	−	+		± C			+	⊥
M H37	+ X	+ X	− X	−	−	− C	−	+	+	−
Soil *M*	+ C	−	+ C	+	+	+ C	+	+	+	⊥

+ = increased O₂ uptake, − = no increased O₂ uptake, C = utilized as sole C sou ???,
X = not utilized as sole C source

droxybenzoic acid stage It also can be shown that when *m*-hydroxybenzoic a? ?
is used as the sole carbon source its oxidation becomes less sensitive to strep??-
mycin, whereas that of benzoic acid has the same sensitivity as the contr?!
Similar experiments were done with *M* 607, *M* BCG, and *M leprae* 4244, gro??
in modified Dubos medium containing benzoic acid The results are compar?!?
to those obtained with *M lacticola*, although the differences are not so gre??
Table 3 summarizes these facts It should be noted that in all cases benzo?? ?
at the beginning is oxidized more rapidly by the strains grown in it than by th?
controls Parallel experiments on the effect of streptomycin on the gro???h ?
the benzoic acid and the control strains were not so clear-cut In a g?ven c??
centration of streptomycin the benzoic acid strains grew out more rapid! f ?
day or two but were then overtaken by the control strains It is po? it? ???
the benzoic acid strains have lost their ability to alter their metab?? ???
readily as the controls
The organisms grown in benzoic acid contain a dark pigment, and th?? c????
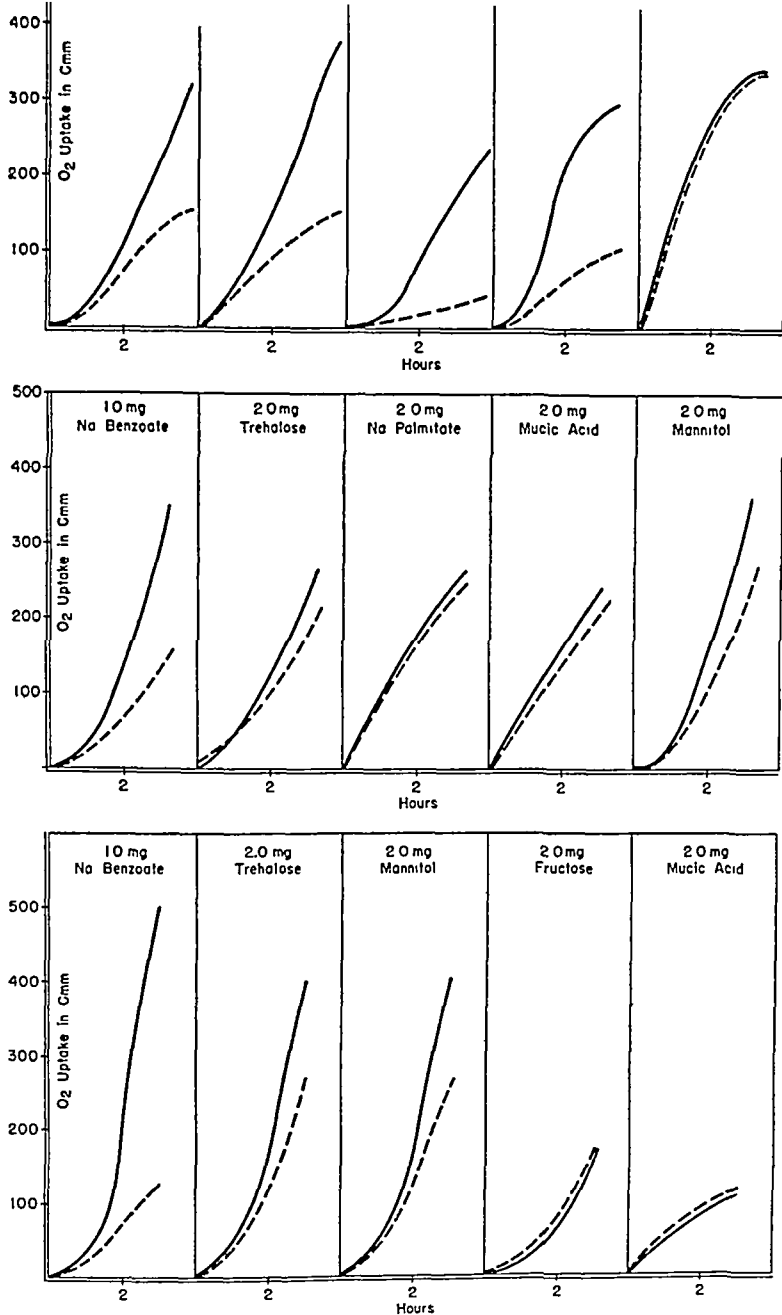
FIG 2 *Top* Effect of 100 µg per ml of streptomycin on the oxidation of various compounds by the soil mycobacterium *Center* Effect of 5 µg per ml of streptomycin on the oxidation of various compounds by *M* BCG *Bottom* Effect of 5 µg per ml of streptomycin on the oxidation of various compounds by *M* 607
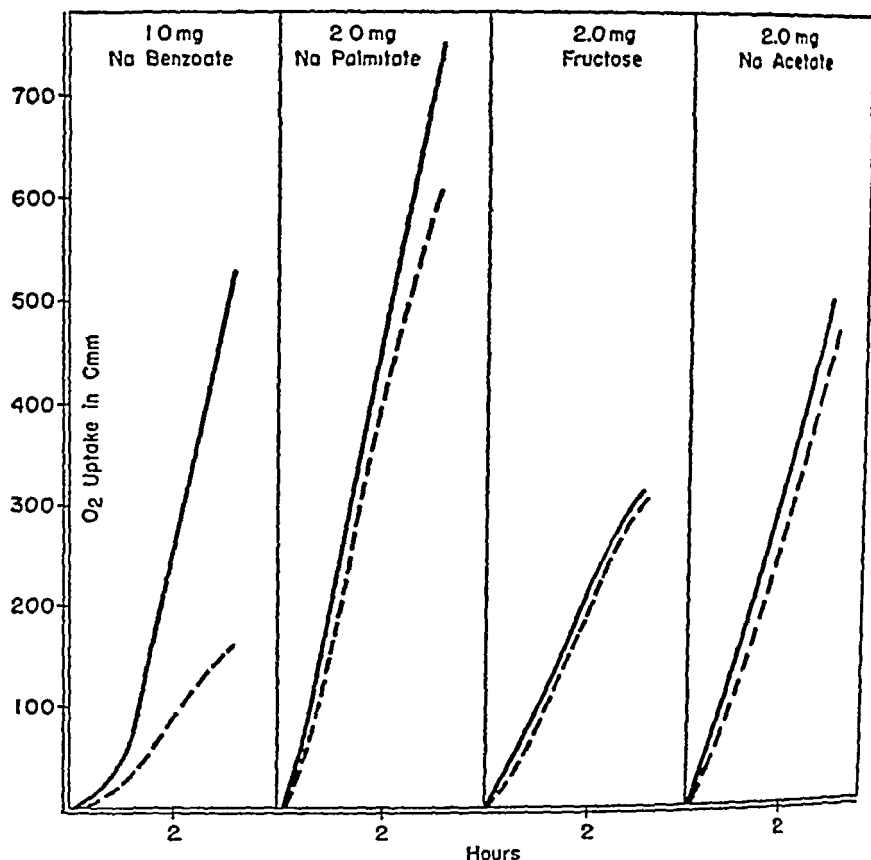
FIG 3 EFFECT OF 5 µG PER ML OF STREPTOMYCIN ON THE OXIDATION OF VARIOUS
COMPOUNDS BY M STERCORIS

TABLE 2

Effect of streptomycin on benzoate oxidation of normal and resistant strains of mycobacteria
Percentage of Inhibition of Benzoate Oxidation

EXPERIMENT	M TUBERCULOSIS NO 607 (5.0 µG/ML STREPTOMYCIN)				
	Normal	R 125 µg %	R 250 µg %	R 500 µg %	R 10 000 µg %
1	64			31	
2	81	90	54	29	
3	85		62		
4	76	59	56	47	
5	74	57	52	26	
6	85				0
	SOIL MYCOBACTERIUM (100 µG/ML STREPTOMYCIN)				
	Normal	P 300 µg %	R 600 µg %	R 1,200 µg %	
1	57	53	11	0	
2	50	25	0	0	
3	64	61	19	16	
4	60	53	22		

ever, that in comparing the oxidation of the benzoic acid and control strains the same amounts of bacteria were always used   This is important because the percentage of inhibition by streptomycin is proportional to the number of organisms present   Thus with M 607 a 29 per cent inhibition of the oxidation of benzoic acid was obtained when 0 5 ml of the bacterial suspension was used, whereas a

TABLE 3

The inhibition of streptomycin of the oxidation of benzoic and m-hydroxide acid by various mycobacteria grown with and without benzoic or m-hydroxybenzoic acid in the medium

(The oxygen uptake of the organisms without added benzoic acid has been subtracted in each case)

	M 607, CONTROL			M 607 GROWN WITH BENZOATE			M LEPRAE 4244 CONTROL			M LEPRAE 4244 GROWN WITH BENZOATE		
Time	Ben zoate	Ben-zoate + 5 µg/ml S	I	Ben zoate	Ben zoate + 5 µg/ml S	I	Ben zoate	Ben zoate + 5 µg/ml S	I	Ben zoate	Ben-zoate + 5 µg/ml S	I
hr	mm³	mm³	%	mm³	mm³	%	mm³	mm³	%	mm³	mm³	%
0 5	7	10	0	15	15	0	0	2	0	11	10	0
1 0	33	26	21	56	47	16	7	5	28	25	22	12
2 0	156	67	57	165	131	21	30	10	66	42	35	17
3 0	352	107	70	297	222	25	60	15	75	56	48	14
4 0	525	141	73	398	289	29	112	18	84	80	64	20
5 0	539	175	69	493	349	29						

	M LACTICOLA CONTROL						M LACTICOLA GROWN ON BENZOATE AS SOLE C SOURCE						M LACTICOLA GROWN WITH m-HYDROXYBENZOATE AS SOLE C SOURCE					
Time	Ben zoate	Ben zoate + 100 µg/ml S	I	m-Hy droxy ben zoate	m-Hy droxy ben zoate + 100 µg/ml S	I	Ben zoate	Ben zoate + 100 µg/ml S	I	m-Hy droxy ben zoate	m-Hy droxy ben zoate + 100 µg/ml S	I	m-Hy droxy ben zoate	m-Hy droxy-ben zoate + 100 µg/ml S	I
hr	mm³	mm³	%	mm³	mm³	%	mm³	mm³	%	mm³	mm³	%	mm³	mm³	%
0 5	0	0	0	0	0	0	27	46	0	15	13	0	55	57	0
1 0	0	0	0	0	0	0	95	110	0	57	32	44	90	94	0
2 0	70	29	59	95	55	42	254	239	6	168	66	61	177	186	0
3 0	163	47	71	187	90	52	378	322	15	242	97	59	317	335	0
4 0	242	60	75	249	117	53	459	368	20	282	117	59			

I = inhibition, S = streptomycin

52 per cent inhibition occurred with 0 25 ml   In this connection it may also be mentioned that the resting respiration is never inhibited by streptomycin

There is always a latent period before the inhibition of the oxidation of benzoic acid takes effect   This may be due to a slow penetration of streptomycin into the cell or to the fact that the drug is not inhibiting the first step in the oxidation of benzoic acid but some intermediate step   Streptomycin was therefore added to the bacterial suspension at different intervals during the oxidation of benzoic

acid, and the time was measured for the attainment of a given percentage of inhibition   The time was the same whether the streptomycin was added before the benzoic acid or halfway through the oxidation process, and this indicates that the latent period is due to the time necessary for the drug to penetrate to its site of action   It also indicates that it is the oxidation of benzoic acid which is inhibited, but does not rule out the possibility that the oxidation of intermediates is also affected

Attempts were made to determine the effect of benzoic acid concentrations on the inhibition by streptomycin, to see whether the two substances are competing for the enzyme surface   In order to do this effectively it is necessary to use relatively large concentrations of benzoic acid so that its concentration is not materially altered by the amount metabolized   It was not possible to use such concentrations as they tended to be inhibitory   Consequently, the percentage of inhibition was measured when 1 0 and 2 0 mg of benzoic acid were added and a constant amount of streptomycin was used   The first significant figures were taken after the latent period when 20 per cent or less of the benzoic acid had been oxidized   Under these conditions the following percentages of inhibition were obtained for 1 0 and 2 0 mg benzoic acid, respectively, and 5 0 μg per ml streptomycin 38 and 25 per cent for M $avium$, 25 and 12 per cent for M BCG   As the oxidation proceeds the percentages of inhibition increase and become the same for the two concentrations of benzoic acid   The indication is, therefore, that the substrate and inhibitor are competing for the enzyme

None of the species completely oxidize benzoic acid to CO_2 and H_2O Usually the oxidation stops when a half of the theoretical amount of oxygen is taken up The end product has not been identified

DISCUSSION

A bacteriostatic agent may act by inhibiting anabolic reactions directly, thus limiting growth by interfering with the synthesis of cellular material, or indirectly by inhibiting oxidative reactions which provide the energy for such synthe   It is evident that certain nonpathogenic mycobacteria can utilize benzoic acid a source of energy and that this reaction is readily inhibited by streptomycin Since there is, moreover, a parallelism between the ability of the drug to inhibit this oxidation and the growth of the organism, the evidence indicates that it may be an important mechanism in the bacteriostatic action of streptomycin It is undoubtedly not the only mechanism   The growth of pathogenic mycobacteria which do not oxidize benzoic acid is equally well inhibited by the drug, and this is also true of a number of gram-negative bacteria that do not utilize compound   It would seem, therefore, that there must be some general reaction which is inhibited in these organisms by streptomycin and that the inhibition of oxidative reactions is secondary   It is of interest, however, that a specific oxidation can be inhibited by a so-called antibiotic agent

SUMMARY

A number of nonpathogenic mycobacteria oxidize benzoic acid   The reaction is inhibited by very small amounts of streptomycin

As the growth of the organisms becomes resistant to streptomycin, so does the oxidation of benzoic acid

The oxidation of benzoic acid by mycobacteria grown in benzoic acid as the sole carbon source or grown in media containing benzoic acid is more resistant to the inhibiting action of streptomycin

Streptamine and streptidine which do not inhibit growth do not inhibit the oxidation of benzoic acid

Benzoic and salicylic acid stimulate the oxygen uptake of pathogenic mycobacteria but are not oxidized by them   This reaction is not inhibited by streptomycin

Other properties of the action of streptomycin on the oxidation of benzoic acid are described

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In certain recent papers Pijper (1946, 1947) has proposed the thesis that bacterial flagella are not origins of locomotion but are artifacts produced on the drying of a carbohydrate envelope surrounding the cell   He regards this as the explanation of much disagreement which has occurred in the literature about the type of flagellation shown by any given species and insists that since flagella are mere artifacts then arrangement around the cell is of no significance   Some time ago one of the present authors (Conn, 1938) proposed an entirely different theory to account for the bacterial species that were declared peritrichic by some students, monotrichic by others, namely, that some species are neither constantly peritrichic nor truly monotrichic, but that they show 'degenerate peritrichic flagellation," some strains having only one flagellum, others two or three flagella, but never a tuft or two or three at one pole

The present investigation was undertaken partly in the hope that the electron microscope would shed some light on the subject and partly with the idea of using Pijper's technique on a different motile organism

CULTURES SELECTED FOR STUDY

When the idea of degenerate peritrichic flagellation was advanced, it was indicated as being especially well represented by species of the genera *Agrobacterium*, *Rhizobium*, and *Chromobacterium*   Accordingly, for the present electron microscope study representatives of these genera were selected as follows   *Agrobacterium tumefaciens* (Smith and Townsend) Conn (the type species of the genus), *Agrobacterium radiobacter* (Beijerinck and Van Delden) Conn, *Agrobacterium rhizogenes* (Rikei et al) Conn, strains of *Rhizobium* from pea, clover, and alfalfa nodules, two strains of *Chromobacterium* spp (violet bacteria, Cruess-Callaghan's nos 17 and 19)

As organisms for use in trying the Pijper technique, *Escherichia coli* and *Bacillus cereus* were selected

TECHNIQUE FOR PREPARING MOUNTS FOR THE ELECTRON MICROSCOPE

It has been our experience, and that of others, that any excessive manipulation of bacterial material in preparing mounts for the electron microscope invariably results in mutilation   Therefore, although a few of the pictures represented here were taken of organisms prepared in the usual way (distilled water preparations

dried on collodion mounted on screens), the majority have been the result of a stripping technique

Williams and Wyckoff (1946) and Schaeffer and Harker (1942) have described techniques for the preparation of true replicas for observation in the electron microscope   Recently Hillier and Baker (1946) have described a technique wherein the top layer of organisms from a young colony was removed in place of an expected replica   In the course of this study hundreds of mounts prepared by the ordinary method were made   In only a few instances have we been able to observe organisms with intact flagella

The method employed in the present work was as follows   The organisms were grown on fresh lima bean agar slopes or agar plates for about 16 hours   A clean microscope slide was then either touched to the growth on the plates and a drop of distilled water added to the adhering organisms, or a loopful of material was transferred to a drop of distilled water on the slide   Bacteria and water were then allowed to stand for 30 minutes   This part of the procedure approaches that recently described by Knaysi et al (1947)   At the end of 30 minutes additional distilled water, enough to flood the slide, was added   The slide was gently rotated a few times and the water poured off, the slide drying in a vertical position

After thoroughly drying, the slides were then shadow-casted with gold, about 8 to 10 Å of gold being deposited on the slide at an angle of 15 degrees   A solution of 0 5 per cent collodion in amyl acetate was allowed to run over the slide, and the slides were again dried in a vertical position   This collodion film was then floated off and mounted on the screens in the usual fashion

Although we expected to obtain shadow-casted replicas, this did not prevail Instead, the organisms and gold film were picked up in toto by the collodion This did not obviate but rather enhanced our preparations   In almost every instance the organisms were observed with intact flagella   Even the slight manipulation employed, however, resulted in detaching some flagella, which could be observed in many fields   The pictures utilized represent organisms that had in most instances no detached flagella in the near vicinity

An RCA type EMC-1 electron microscope was employed

FLAGELLATION OF AGROBACTERIUM SPECIES

The first work on flagellation of A tumefaciens (crown gall organism) done with the electron microscope, part of which has already been published (Braun and Elrod, 1946), seemed to indicate clearly a single polar flagellum (figure 1, no 1)   Later it came to be noticed that the flagellum was not always attached exactly at the pole (figure 1, nos 2, 4, and 5), a circumstance previously stated (Conn, Wolfe, and Ford, 1940) to indicate degenerate peritrichic flagellation

Preparations made at about the same time from A rhizogenes (cause of hairy root) and A radiobacter also showed a decided predominance of cells with single flagella, usually at the pole (figure 1, nos 3, 6)   These organisms like A tumefaciens have been described, by some authors at least, as having peritrichous flagella
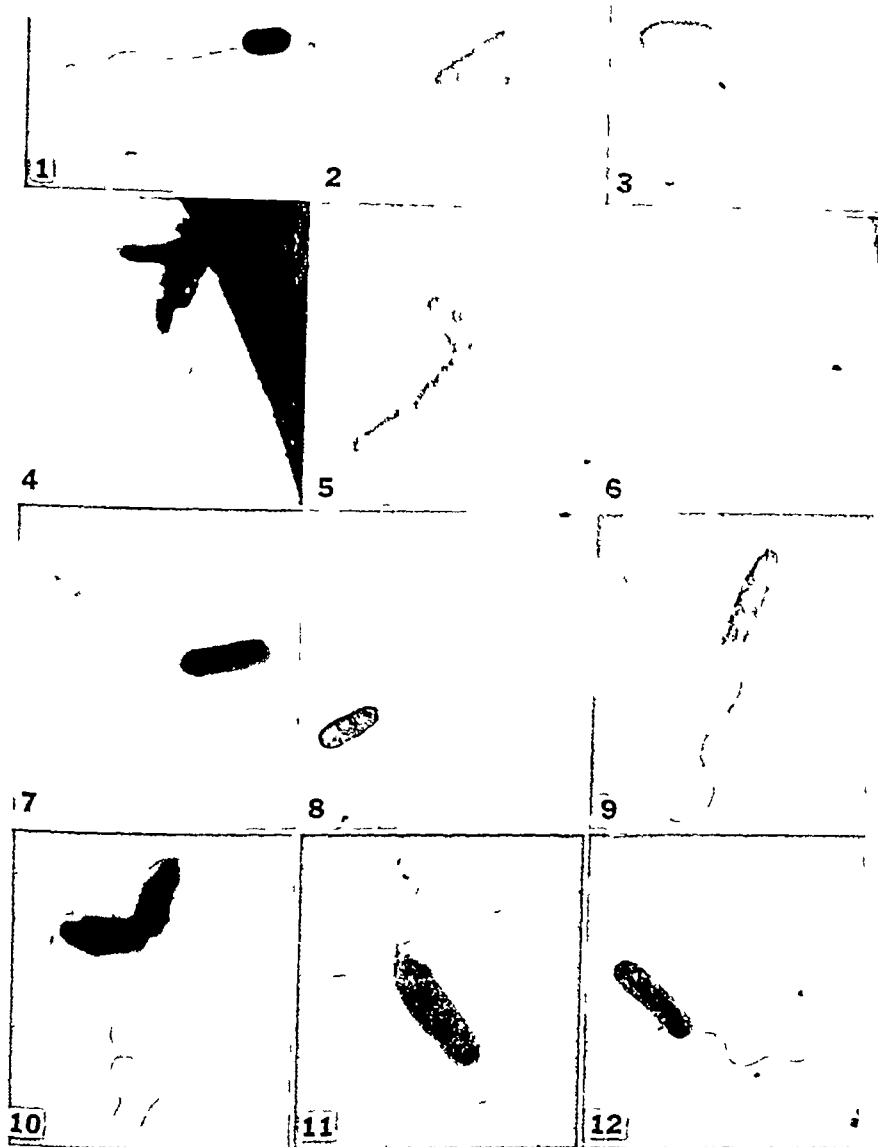
FIG 1 ELECTRON MICROGRAPHS (GOLD SHADOWED) SHOWING FLAGELLA
Nos 1, 2, 4, and 5 *Agrobacterium tumefaciens*
No 3 *Agrobacterium rhizogenes*
No 6 *Agrobacterium radiobacter*
Nos 7 and 8 Cruess-Callaghan's culture no 19, "*Bacillus violaceus*"
No 9 Cruess-Callaghan's culture no 17, "*Bacillus membranaceus amethysticus*"
No 10 *Rhizobium* culture from pea nodule
No 11 *Rhizobium* from clover nodule
No 12 *Rhizobium* from alfalfa nodule

As the same situation was found in the case of the violet *Chromobacterium* species (figure 1, nos 7, 8, 9), it was decided to make a more intensive study of certain other organisms (the legume nodule bacteria) thought to have the same type of flagellation

FLAGELLATION OF RHIZOBIUM

The first micrographs of *Rhizobium* species were made on single strains of the clover, pea, and alfalfa nodule organisms and are shown in figure 1, nos 10 to 12 These micrographs were made after numerous fields were examined visually and are regarded as entirely typical of the majority of organisms present   The impression to be gained from these preparations is clearly that the clover and alfalfa cultures each have a single polar flagellum, whereas the pea organism may be peritrichic   Now these species belong to the group of nodule bacteria which are generally recognized as peritrichic (the soybean and the cowpea organisms, on the other hand, being regarded as monotrichic)   The alfalfa organism, in particular, has so generally been accepted as peritrichic, and so many photomicrographs indicating as much have been published, that these results seemed to call for further study

Accordingly a collection of 12 strains of the alfalfa organism was obtained from Dr A W Hofer of Geneva, New York, and they were studied by essentially the same technique   Electron micrographs (one of each strain) are shown in figure 2   It is clearly seen that some cells are monotrichic, others peritrichic   In every instance typical cells were selected, after considerable search of each preparation, before the micrograph was made   In other words, some strains seemed to show one type of flagellation, others the other, that is, there are both monotrichic and peritrichic strains of this species

Apparently, therefore, the electron microscope bears out in regard to the *Rhi zobium* species the same conclusion that had been drawn from stained preparations, namely, that peritrichic and monotrichic strains may occur in the same species, probably monotrichic and peritrichic cells in the same strain   Obviously, in the case of such organisms as this, the type of flagellation cannot be employed as a criterion for species diagnosis   This conclusion does not, however, invalidate type of flagellation as a diagnostic feature in the case of truly peritrichic species (as in the colon-typhoid group) or definitely lophotrichic forms (like *Pseudomonas fluorescens*)

Present work with the electron microscope on the other organisms discussed above has not included a sufficient number of strains to show whether the same conclusion could be drawn concerning them   The presumption is that such would be the case   If that is true, it seems logical enough to explain observed discrepancies on the basis of degenerate peritrichic flagellation

SIGNIFICANCE OF FLAGELLATION

Piper's explanation of such discrepancies as due to flagella being artifacts not concerned in motility seems harder to accept   There are several argument against this theory

faciens   J Bact , **52,** 695–702

CONN, H J , AND WOLFE, GLADYS E   1938   The flagellation of bacteria   Science, **87,** 283–284

CONN, H J , WOLFE, GLADYS E , AND FORD, MARK   1940   Taxonomic relationship of *Alcaligenes* spp   to certain soil saprophytes and plant parasites   J Bact , **39,** 207–226

HILLIER, JAMES, AND BAKER, R F   1946   The mounting of bacteria for electron microscope examination   J Bact , **52,** 411–416

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WILLIAMS, R C , AND WICKOFF, R W G   1946   Applications of metallic shadow-casting to microscopy   J Applied Phys , **17,** 23–33

SUBMERGED CULTURE INVESTIGATIONS

HARRY HUMFELD

Western Regional Research Laboratory,[1] *Albany, California*

Received for publication August 1, 1947

A small laboratory fermentor which employs mechanical agitation for the dispersion of air introduced under pressure has previously been described by Feustel and Humfeld (1946) This fermentor has an operating capacity of 500 to 2,000 ml and has been found very useful for small-scale, yeast-culturing investigations, as well as for studies on the production of subtilin activity by *Bacillus subtilis* (1947)

Certain limitations, however, principally those of capacity and effectiveness of foam breaking, have led to the development of an improved fermentor having a larger capacity and a more effective mechanical foam breaker This fermentor also has a stirring device, which is so designed that air for the aeration of the culture liquid can be drawn in from the atmosphere by suction created behind the stirring blades, as compared with air introduced under pressure This paper describes the improved fermentor and some of the preliminary results obtained

DESCRIPTION OF THE FERMENTOR

The fermentor vessel consists of a standard pyrex glass jar, 12 inches in diameter and 24 inches high This jar is fitted with a gasketed, stainless-steel cover The stainless-steel agitation-aeration assembly shown in figure 1 is suspended from the cover and is inserted in the pyrex jar

Agitation and aeration are accomplished by means of a special air-dispersing device, mounted at the lower end of the stirring shaft near the bottom of the fermentor Two adjustable truncated cones $3\frac{1}{4}$ inches in diameter are mounted on the shaft above the agitation-aeration device By inverting these cones the stirring characteristics may be changed Four metal struts are attached at right angles to the cover at equidistant points around the periphery of the cover, approximately 1 inch from the edge A metal web fastened to the lower end of these struts furnishes rigidity and support for the stirring shaft

The agitation-aeration device consists of a short, central, hollow cylinder, to which four sets of tubes are fastened These tubes are bent in the form of arcs at right angles to the cylinder A small vane is attached to each alternate set of tubes This device rotates between two plates The upper plate is attached to the supporting web described above The lower plate is attached to, and kept parallel with, the upper plate by studs The lower plate has a large circular hole, through which the culture liquid enters An air-intake pipe extends from the cover to a point directly below the hollow, central core of the agitation-

aeration device   As the turbine device rotates, the suction created behind the vanes at the end of the tubes draws air through the air-intake pipe into the central, hollow cylinder, from which it is conducted into the culture liquid by the radiating tubes   The height of the intake pipe is adjustable at the cover, so that the amount of air drawn in can be regulated   The maximum amount of air is drawn in with the lower opening of the intake pipe raised as high as possible without touching the revolving hollow cylinder of the turbine   Lowering the intake pipe decreases the air flow

FIG 1   FERMENTOR ASSEMBLY FOR LARGE LABORATORY FERMENTOR

The foam-breaking device consists essentially of a disk mounted on the shaft just below a larger fixed cone   The disk, about 8 inches in diameter, is provided with an interior set of vanes and an exterior set of vanes, half of the vanes of each set are turned up and half are turned down   The outer edge of the cone extends just beyond the interior set of vanes   The inner set of vanes scoop up the foam as it rises to the height of the disk, the centrifugal force created by the rotation throws the foam against the inner surface of the cone, which, in turn forces it out onto the upper surface of the disk, whose outer set of vanes di

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aeration device As the turbine device rotates, the suction created behind the vanes at the end of the tubes draws air through the air-intake pipe into the central, hollow cylinder, from which it is conducted into the culture liquid by the radiating tubes The height of the intake pipe is adjustable at the cover, so that the amount of air drawn in can be regulated The maximum amount of air is drawn in with the lower opening of the intake pipe raised as high as possible without touching the revolving hollow cylinder of the turbine Lowering the intake pipe decreases the air flow

Fig 1 Fermentor Assembly for Large Laboratory Fermentor

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operation t e form is broken effectively, and the liquid is returned down the inside wall of the vessel to the culture liquid

The fermentor is equipped with a set of pH meter electrodes, the leads of which pass through removable waterproof tubes to the pH meter, hence, the pH of the culture may be determined at any instant   A thermometer inserted through a small well provides for the reading of the temperature of the culture at any time The fermentor is also provided with a sampling tube, by means of which a sample may be drawn periodically for analyses   The power for operation of the fermentor is furnished by a $\frac{1}{4}$-hp ball-bearing, variable-speed, electric motor, mounted vertically at the center of the cover and fastened to the stirring shaft by a self-aligning coupling

The entire fermentor assembly may be taken apart readily for cleaning and replacement and adjustment of parts   It may be assembled without the motor for sterilization   The operating capacity of the fermentor ranges from 10 to 18 liters of culture medium [2]

OPERATION OF THE FERMENTOR

The fermentor has been found suitable for the submerged culturing of aerobic microorganisms   It has been employed in the propagation of yeast, in which case sterilization of the media and of the equipment is not essential, as well as for the production of antibiotics   For the latter purpose the equipment and the culture medium are sterilized, since the maintenance of pure culture is usually essential   Except for the details of sterilization and care necessary for keeping the culture free of contamination, the technique of operation in all cases is essentially identical   The air drawn in is sterilized by passage through a previously sterilized tube loosely packed with glass wool

The inoculated medium is transferred to the fermentor, the motor is placed in position on the cover, and the motor shaft and stirrer shaft are connected by means of the self-aligning coupling   The motor is started and the amount of air drawn in is regulated by adjusting the height of the air-inlet pipe by means of the adjusting screw on the fermentor cover   The air flow also may be controlled by adjusting the speed of the motor, which, of course, simultaneously changes the rate of stirring   For most operations a ratio of one volume of air per minute per volume of culture medium is satisfactory   By reducing the distance between the lower tip of the inlet tube and the hollow bore of the agitation-aeration unit, volumes of air as high as two and one-half times the volume of the culture medium may be drawn in per minute   As the volume of growth in the medium increases during the fermentation, the viscosity of the culture suspension gradually increases, hence, if it is desired to maintain a uniform rate of air flow, it is necessary to readjust the distance between the core and the inlet pipe   A record of the pH of the medium and its temperature is kept, and if it is desired to control the pH, a suitable amount of base or acid solution is added as may be required

[2] Detailed engineering drawings for the construction of similar units may be obtained from the Western Regional Research Laboratory, Albany, California

Since the fermentation usually generates considerable heat, it has been found desirable to place the fermentor in a suitable water bath and to hold the water in the bath at such a temperature as will maintain that desired in the fermentor By means of the sampling tube samples are withdrawn periodically for determinations of cell volume, nutrient concentrations and, in the case of the production of antibiotics, for the bioassay purposes The fermentor in operation is shown in figure 2

FIG 2 LARGE LABORATORY FERMENTOR FOR SUBMERGED CULTURE INVESTIGATIONS IN OPERATION

FERMENTATIONS

Yeast production An example of the use of the fermentor for yeast production is presented here The yeast used in this run was *Torulopsis utilis* (ARRL Y-900) The medium was made from pear juice concentrate from cannery pear waste One liter of the concentrate, which contained 26 per cent sugar, was diluted initially with 12 liters of water The mineral salts required—15 4 grams of 85 per cent phosphoric acid, 3 6 grams of potassium sulfate, and 1 0 gram of magnesium sulfate—were added

The inoculum was prepared by transferring a 10-ml suspension from the growth of a stock culture slant to the surface of a shallow layer of wort agar in two Fernbach flasks The flasks were incubated for 24 hours at 30 C The growth on the agar was suspended in a small amount of the pear juice medium and, with the rest of the medium, transferred to the fermentor The motor was operated at

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After the sugar in the pear juice medium at the start of the fermentation had been utilized, additional full-strength concentrate was added from time to time The details of operation and the yield of yeast obtained are given in table 1

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<div align="center">

TABLE 1

Results of a yeast propagation experiment

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TIME	pH	YEAST VOLUME	VOLUME IN FERMENTOR	PEAR JUICE CONCENTRATE ADDED	CONC NH₄OH ADDED	AIR	WEIGHT OF YEAST PRODUCED (DRY BASIS)
hr		$\%$	$liters$	$liters$	ml	$liters/min$	g
			12 H_2O	1 c	27		
0	6 4	0 10	13	—	—	16 5	2 9
4	6 2	0 50	13	—	—	16 5	14 3
6	5 0	1 00	13	—	—	16 5	28 6
$7\frac{1}{2}$	3 3	3 00	13	—	30	16 5	94 4
9	5 1	5 8	13	—	35	16 5	166
$9\frac{3}{4}$	4 2	6 5	13	—	—	16 5	186
$10\frac{1}{2}$	3 5	7 5	14	1	55	13 5	231
$11\frac{1}{2}$	3 9	10 5	14	—	40	13 5	323
$12\frac{1}{2}$	3 6	11 2	15	1	50	11 5	370
$13\frac{1}{2}$	5 2	13 0	15	—	25	11 5	429

Total sugar supplied	780 g
Dry yeast produced	429 g
Yield of yeast (based on sugar supplied)	55%
Increase of yeast over inoculum	148X
Average generation time	112 min
Number of generations	7 23

in the autoclave for 1 hour at 15 pounds' steam pressure The procedure was identical with that described by Stubbs *et al* (1947) for the production of subtilin in small fermentors The medium was made by diluting 1,400 grams of an asparagus juice concentrate (70 per cent total solids) to 14 liters This medium was distributed in 3 5-liter portions in 4-liter bottles and sterilized for 30 minutes at 100 C in the autoclave When cool the reaction of the medium was adjusted aseptically from about pH 5 5 to pH 7 by addition of the required amount of 10 N NaOH, then transferred aseptically to the fermentor, and the inoculum added

The inoculum was made by suspending the growth from an agar slant in a small amount of medium and adding this suspension to 500-ml portions of the asparagus juice medium in each of two Fernbach flasks These flasks were incubated

for 24 hours at 35 C, then the contents were transferred to a sterile Waring "blendor," and the bacterial pellicle was thoroughly broken up and suspended in the medium   This suspension was added to the sterile medium in the fermentor

The initial motor speed was 1,300 rpm, and the aeration was 14 liters of air per minute   As the bacterial cells in the medium multiplied, the medium became more viscous and had a tendency to decrease the rate of aeration   The rpm were gradually increased until 1,600 rpm were attained, thus the rate of aeration was kept uniform   The pH at the start was 6 9 and gradually dropped

Fig 3   Growth of Bacillus subtilis and Subtilin Production

to 6 15 in 5 hours, after which it gradually increased to 7 45 at 8 hours, when maximum cell volume had been obtained   The culture medium was harvested after 8½ hours' incubation at 35 C

The bioassay for subtilin activity was made according to the method described by Lewis et al (1947)   The volume of the centrifuged cells calculated as percentage of the culture medium and the subtilin activity calculated as milligrams of subtilin per liter are shown in figure 3

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FIG 3   GROWTH OF BACILLUS SUBTILIS AND SUBTILIN PRODUCTION

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CHARACTERISTICS OF LEUCONOSTOC MESENTEROIDES FROM CANE JUICE

C S McCLESKEY, L W FAVILLE, and REX O BARNETT

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The bacteria which produce gum or slime in sugar solutions are present in large numbers in the sugar-cane juice of sugar factories and not infrequently cause trouble in the factory processes   These organisms, together with other related forms producing levo-rotatory lactic acid, relatively large amounts of volatile acid, carbon dioxide, and ethyl alcohol from glucose, have been allocated to the genus *Leuconostoc* Van Tieghem by Hucker and Pederson (1930)   The species commonly encountered in cane juice is *Leuconostoc mesenteroides* according to the characterization of that species by Hucker and Pederson that was adopted by Bergey *et al* (1939)

Since this species is known to be rather heterogeneous, both serologically (Hucker, 1932) and biochemically (Hucker and Pederson, 1930), and since this organism has been suggested as a possibility for use in the biological assay of certain amino acids (Dunn *et al*, 1944, Horn, Jones, and Blum, 1947) and vitamins (Gaines and Stahly, 1943), an investigation of the diversity of strains in this species is desirable   This report is limited to a study of strains isolated from cane juice at the Experimental Sugar Factory of the Louisiana State University

The older literature pertaining to the organisms of this group has been adequately reviewed by Hucker and Pederson (1930) in their classical work, which demonstrated the essential similarity of variously named cultures isolated from vegetable products, dairy products, and sugar solutions   More recently Niven, Smiley, and Sherman (1941) have pointed out that *Streptococcus salivarius* produces a considerable amount of slime from sucrose, in this way resembling *Leuconostoc*   Also Niven, Kiziuta, and White (1946), White and Niven (1946), and Niven and White (1946) observed that many isolates from cases of subacute bacterial endocarditis produced gum in liquid sucrose media, a characteristic suggestive of the *Leuconostoc* group   The serological relationship of the *Leuconostoc* polysaccharide to that of the type II pneumococcus has been studied by Neill and coworkers (1941)

In this study isolations of *Leuconostoc* strains were made from sugar-house cane juice plated on a medium of the following composition

Difco tryptone	10 g
Difco yeast extract	5 g
Raw sugar	100 g
Difco agar	20 g
Distilled water	1,000 ml
pH 6 7 Sterilized 15 minutes at 121 C	

Most of the strains included in this study were isolated from cane juice which had been frozen and stored several months at about −18 C   The same general types were found also in the fresh juice, but possibly not in the same relative frequency   Care was exercised that all the different colony types of gum formers were isolated for study, and 740 cultures were obtained

It was noted that there were four distinct types of gum-forming colonies, and the primary grouping of isolates was based on colonial characteristics   These types differed in size, elevation, topography, and optical characters, and were designated A, B, D, and F by Faville (1947)   Descriptions of these colonies on

TABLE 1

Morphological and colonial characteristics of gum-forming organisms

MORPHOLOGICAL CHARACTERISTICS (2% RAW SUGAR BROTH)	COLONY TYPES			
	A	B	D	F
Shape	Oval, spherical	Oval, spherical	Oval, spherical	Oval, spherical
Size	0 5–0 7 by 0 7–1 0 μ	0 7–0 9 by 0 7–1 2 μ	0 5–0 7 by 0 7–1 0 μ	0 5–0 7 by 0 7–1 0 μ
Grouping	Occur in large clusters and pairs, few chains	Occur in pairs and occasional chains of 4–6 cells	Occur in pairs and occasional chains of 4–6 cells	Occur in pairs and occasional long chains of 20–30 cells
COLONIAL CHARACTERISTICS (2% RAW SUGAR AGAR)				
Form	Circular	Circular	Circular	Circular
Elevation	Convex	Conical	Hemispherical	Hemispherical
Height of colony	1 mm or less	3–4 mm	4–8 mm	3–4 mm
Diameter of colony	3–4 mm	4–6 mm	8–12 mm	4–6 mm
Surface	Smooth	Rugose	Smooth	Smooth
Margin	Entire	Entire	Entire	Entire
Density	Semitransparent	Opaque	Transparent	Opaque

10 per cent raw sugar are presented in table 1   Photographs of these types are shown in figures 1 and 2

The composition of the medium and temperature of incubation had a pronounced effect on the colonial appearance of these cultures   Incubation at 20 to 25 C on 10 per cent sucrose or raw sugar agar was most satisfactory for showing group differences   When grown at 37 C colonies of all types were smaller, and there was much less evidence of gum formation   Colonies of types A and D showed little resemblance to those of the same type grown at 20 to 25 C   At the lower temperature both A and D colonies "dripped" down onto the lid of the inverted petri dish, whereas at 37 C the colonies were small, opaque, and nearly flat   The colonies were most characteristic after incubation for 3 to 5 days at room temperature   Most strains of the A and D types produced colonies which were so clear that print could be read through them
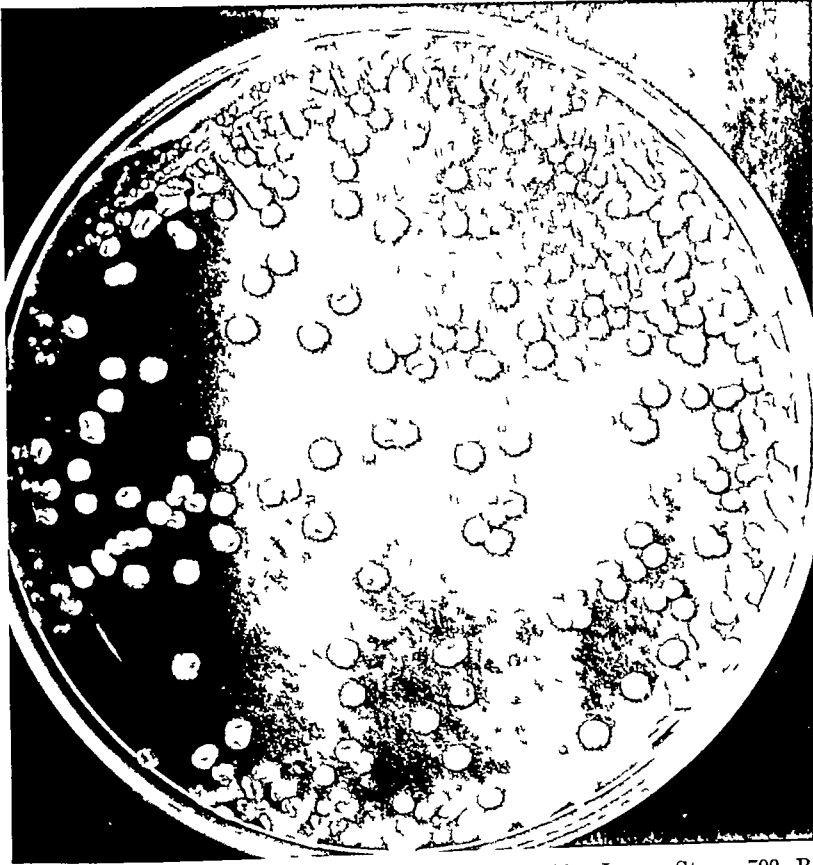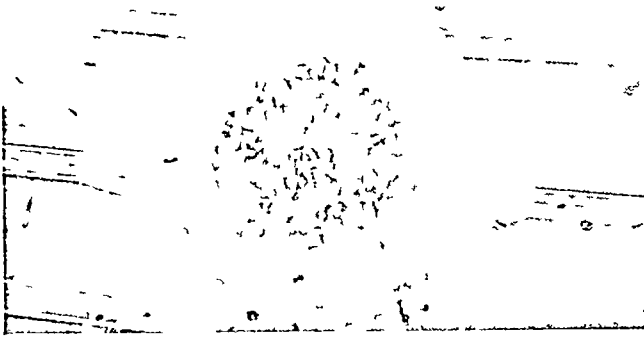
Fig 1 *Upper* Strain 730, A type, 1 day culture ×20 *Lower* Strain 700, B type
Culture on 10% raw sugar agar, incubated at room temperature for 3 days Colonies are
conical, rugose, and cartilaginous Some strains form a clear, colorless watery gum at the
base of the colony
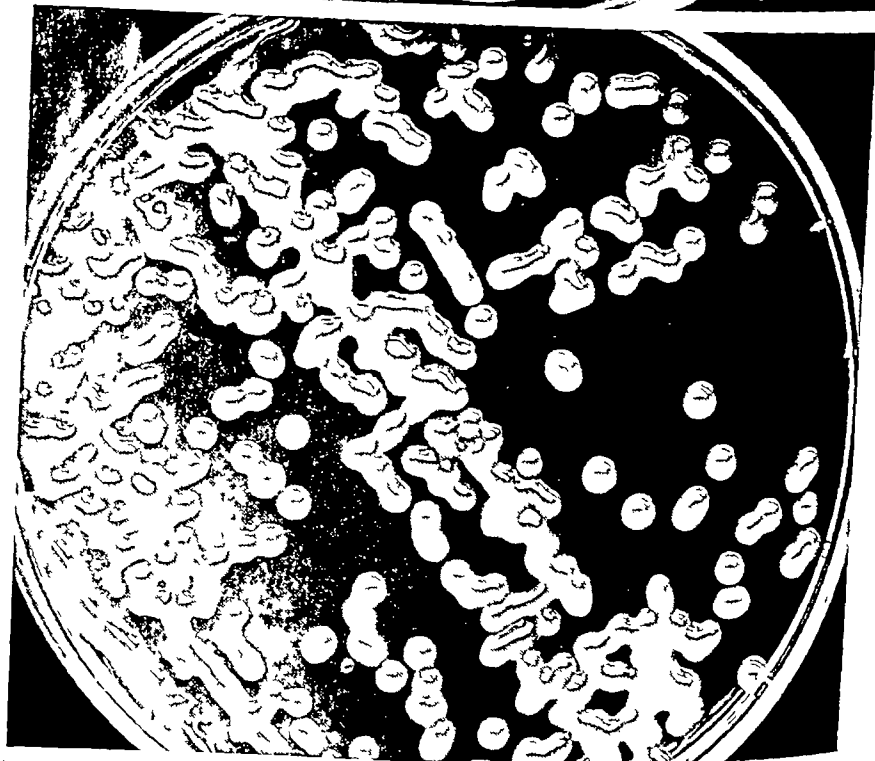
FIG 2 *Upper* Strain 200, D type   *Lower* Strain 860, F type   Three day culture on 10% raw sugar agar, incubated at room temperature

700

TABLE 2

Fermentation reactions

COLONIAL TYPES AND PERCENTAGE OF STRAINS FERMENTING TEST SUBSTANCES			
A(18 strains)	B(23 strains)	D(112 strains)	F(15 strains)
100	100	97*	100
100	100	45	100
100	100	100	100
100	100	100	100
100	100	100	100
100	100	100	100
100	100	100*	100
100	100	40	33
100	100	100	100
100	100	71	100*
100	100	46	0
100	100	80	33
0	4	32	0
100*	100*	40	0
0	4	50*	0
0	4	50*	0
0	0	0	0
0	0	0	0
0	0	0	0
100	100	94	100
100	100	72	100
100	96	51	20
0	0	0	0
0	0	0	0
100*	80*	10*	100*
0	0	0	0
0	0	0	0
0	0	0	0

n by some or all strains

e selected for more detailed study   All isolates
vere alike in the following characters

limethylaniline monohydrochloride test)

nod) negative (5 days)
y nutrient agar or broth
ite, and citrate not utilized as sole carbon source
 layed acid formation, few strains produced coagula-

of 4 per cent but not in 6 per cent NaCl

Immediately after isolation all strains were tested for ability to ferment sucrose, lactose, xylose, and arabinose   At that time a considerable number of D
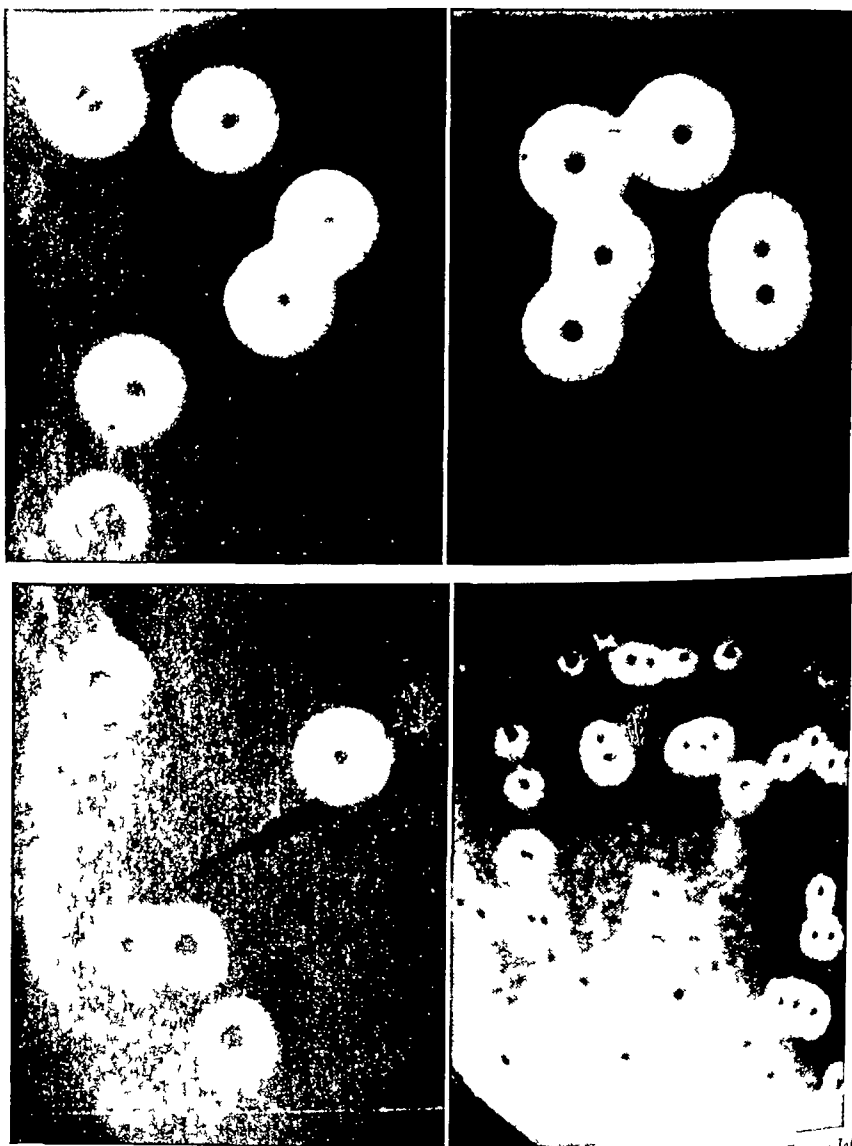
FIG 4 *Upper left* Strain 730, A type   *Upper right* Strain 158, B type   *Lower left* Strain 835, D type   *Lower right* Strain 714, F type   Colonies on sheep's blood agar with 0 1% added glucose, incubated at room temperature for 7 days   × 7   Only the den e central portion of the colonies shows in the figures   The zone of hemolysis was in most cases less than half the diameter of the colony

strains failed to ferment sucrose and lactose, but when tested some months later these strains were found to produce a delayed fermentation   Successive tran-

TABLE 2

Fermentation reactions

SUBSTRATE	COLONIAL TYPES AND PERCENTAGE OF STRAINS FERMENTING TEST SUBSTANCES			
	A(18 strains)	B(23 strains)	D(112 strains)	F(15 strains)
Xylose	100	100	97*	100
Arabinose	100	100	45	100
Glucose	100	100	100	100
Fructose	100	100	100	100
Galactose	100	100	100	100
Mannose	100	100	100	100
Sucrose	100	100	100*	100
Trehalose	100	100	40	33
Maltose	100	100	100	100
Lactose	100	100	71	100*
Melibiose	100	100	46	0
Cellobiose	100	100	80	33
Melezitose	0	4	32	0
Raffinose	100*	100*	40	0
Starch	0	4	50*	0
Dextrin	0	4	50*	0
Glycogen	0	0	0	0
Xylan	0	0	0	0
Inulin	0	0	0	0
Esculin	100	100	94	100
Salicin	100	100	72	100
Amygdalin	100	96	51	20
Glycerol	0	0	0	0
Sorbitol	0	0	0	0
Mannitol	100*	80*	10*	100*
Dulcitol	0	0	0	0
Inositol	0	0	0	0
Erythritol	0	0	0	0

* Indicates delayed fermentation by some or all strains

Of the 740 isolates, 168 were selected for more detailed study   All isolates were gram-positive cocci, and were alike in the following characters

Catalase negative
Oxidase negative (*para*-aminodimethylaniline monohydrochloride test)
Methylene blue reduced
Indole not produced
Nitrates not reduced
Voges-Proskauer (Barrett's method) negative (5 days)
No perceptible growth in ordinary nutrient agar or broth
Acetate, lactate, tartrate, succinate, and citrate not utilized as sole carbon source
Litmus milk unchanged or long-delayed acid formation, few strains produced coagulation even after 28 days
Most strains grew in the presence of 4 per cent but not in 6 per cent NaCl

but no member of the D type produced so much as 10 per cent gas, whereas the maximum for the A, B, and F types (under seal) was 50, 70, and 95 per cent, respectively   The F strains were by far the most active gas formers in sucrose media

The cultivation of the isolates at low pH and at a high temperature revealed further differences among the types (table 6)   At pH 4 1 all isolates grew, but at pH 3 75 none of the A strains and only 2 of the D strains initiated growth At pH 8 5 about half the B strains failed to grow, but practically all isolates of the other types grew   When incubated at 8 to 10 C all strains grew, but most of

TABLE 5

Gas production by Leuconostoc mesenteroides

TYPE	STRAINS	PERCENTAGE OF STRAINS PRODUCING VISIBLE EVIDENCE OF GAS				AVERAGE VOLUME OF GAS (%)*
		Durham fer tubes		15% ucrose no seal	5% sucrose paraffin seal	
		1% sucrose	10% sucrose			
A	18	0	83 4	0	100	24
B	29	3 4	69 0	57	100	23
D	38	0	0	0	97 4	4
F	14	0	100	100	100	37

* Results obtained from tubes with paraffin seal

TABLE 6

Limiting temperature and pH for growth of Le iconostoc mesenteroides

TYPE	STRAINS TESTED	PERCENTAGE OF STRAINS GROWING AT					
		pH 3 75	pH 4 1	pH 8 5	8–10 C	37 C	44 C
A	18	0	100	94 4	100	100	50
B	30	37 5	100	53 1	100	100	0
D	40	5	100	97 5	30*	100	74
F	16	86 6	100	100	100	100	0

All readings were made after incubation for 3 days
* All strains were growing after 9 days

the D isolates developed very slowly and did not show evidence of growth within 3 days   At 44 C (air temperature) B and F strains were inhibited, but many of the A and D strains grew   When tested at 45 C in a water bath, none of the A isolates and only 4 of the D strains grew

The final acidity developed in 15 per cent sucrose broth was found to be greatest at room temperature (25 to 30 C) in all groups (table 7)   This temperature was found to be very favorable for gum production also (table 4), but it is apparent that high acid formation is not always accompanied by the formation of large amounts of gum   Greater viscosity in every instance occurred at 8 to 10 C with relatively low acidity rather than at 37 C with high acidity

Isolates of type D were found to vary quite widely in final acidities produced in

more heterogeneous than the other groups

On sheep's blood agar containing 0 1 per cent added glucose the different groups could not be clearly distinguished   The colonies of types A, B, D, and F were 0 7 to 2 0 mm in diameter, slightly raised, and gray in color   Colonies of the F type were generally somewhat smaller than those of the other types   After 2 to 7 days all A, B, and D strains produced distinct hemolysis, whereas some of the strains of type F produced slight or no hemolysis (figure 4)

SUMMARY

Leuconostoc mesenteroides isolates from cane juice were found to consist chiefly of four relatively distinct colonial types when grown on 10 per cent raw sugar agar at 20 to 28 C   These types were found to differ also in certain fermentation reactions, in amount of gum, gas, and acid produced, and in the temperature and pH requirements for growth

TABLE 7

The effect of temperature on the final pH in 15 per cent sucrose broth

TYPE	STRAINS TESTED	16 DAYS 8–10 C			16 DAYS 25–30 C			10 DAYS 37 C			8 DAYS 44 C		
		Low	High	Avg	Low	High	Avg	Low	High	Avg	Low	High	Avg
A	18	4 05	4 35	4 25	3 61	3 78	3 70	3 70	3 89	3 80	5 25	6 2	5 58
B	28	4 0	4 68	4 27	3 60	3 82	3 68	3 75	4 02	3 89		no growth	
D	38	4 14	5 45	4 77	3 97	4 25	4 12	3 95	4 70	4 15	4 4	5 8	4 87
F	14	4 0	4 30	4 05	3 86	3 95	3 90	3 80	4 05	3 90		no growth	

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in the water bath   The contents of the tubes were then mixed, placed in the refrigerator, and observed the next day   All sera were controlled by testing with the homologous antigen and with a saline control

In table 1 are presented the results of the precipitin tests using a total of 29 extracts   Of type A two extracts were tested against four sera, two were precipitated by 730(A) serum and none by sera 11(D), 548(D), and 860(F)   Five type B extracts were tested of which 2 gave positive reactions with serum 730(A), 1 with serum 11(D), 2 with serum 548(D), and none with serum 860(F)   In the D type 16 extracts were used, of which none was precipitated by serum 730, 2 by serum 11(D), 2 by serum 548(D), and none by serum 860(F)   In the F type

TABLE 1

The precipitin test applied to the differentiation of Leuconostoc mesenteroides strains

TYPE	STRAINS TESTED	PER CENT POSITIVE PRECIPITATION REACTIONS			
		730(A)	11(D)	548(D)	860(F)
A	2	50	0	0	0
B	5	40	20	40	0
D	16	0	12 5	12 5	0
F	6	16 6	16 6	16 6	33 3

TABLE 2

Agglutination reactions in the Leuconostoc mesenteroides group

TYPE	STRAINS TESTED	PER CENT POSITIVE REACTIONS WITH ANTISERUM					
		7 0(A)	158(B)	11(D)	1060(D)	548(D)	850(F)
A	18	100*	0	0	0	0	5 5
B	30	0	6 6	0	0	0	0
D	50	22	10 5†	28	54	34	20
F	17	11 8	23‡	5 9	0	0	94 1

* Percentages are based on agglutination at 1 100 dilution
† Thirty-eight strains tested
‡ Thirteen strains tested

we used 6 extracts, of which 2 were precipitated by serum 860(F) and 1 by each of the other sera (the same extract in each case)

For the agglutination tests the same sera were used   Four different dilutions were employed—1 50, 1 100, 1 200 or 1 400, and 1 600 or 1 1,000   The highest dilution employed was in each case the titer for the homologous organism. The tests were conducted in 0 5 per cent saline to reduce the likelihood of spontaneous agglutination   The antigens were 24- to 48-hour tryptone glucose yeast extract broth cultures

Table 2 shows the results obtained in the 1 100 dilution or higher   Although there was cross agglutination between the four types, there was some evidence that certain types constitute reasonably distinct serological groups   Type A

serum of that type agglutinated only one culture in addition to the homologous organism

The results obtained in agglutinin absorption tests further confirm the serological homogeneity of the type A strains (table 3)   Absorption with the F type

TABLE 3

Agglutinin absorption tests with antiserum 730 (type A)

ANTIGENS		UNABSORBED	ABSORBED WITH 1064(D)	ABSORBED WITH 864(F)
Type A	18 strains	400*	400	400
Type D	1,068	200	0	100
	1,061	100	0	100
	1,063	200	0	0
	1,064	400	0	100
	1,067	200	0	100
	956	200	200	200
	1,065	400	0	100
Type F	860	100	0	0
	861	50	0	0
	864	50	0	0
	1,010	400	100	0

0 Indicates no agglutination
* Numbers indicate highest dilution of serum causing agglutination

TABLE 4

Absorption tests with antiserum 158 (type B)

TYPE	CULTURE NUMBER	158 B NOT ABSORBED	158 B ABSORBED WITH 1064(D)
B	164	400	200
	158	400	400
D	1,061	400	400
	1,064	400	100
	1,067	400	400
	1,068	400	200
F	1,010	100	100
	1,011	100	100
	1,012	100	100

strain failed to remove the agglutinins for the A type but effectively removed the agglutinins for all the F types and partially removed those for the D type as well   Similarly absorption with the D type strain removed none of the A agglutinins but removed the agglutinins for most of the D and F types   Absorption tests with the anti-B serum using a D strain resulted in lowered titer for a B strain and a D strain, with no effect on the homologous B strain and the F strains (table 4)   Likewise absorption of the anti-F serum with a D antigen had little effect on the titer for either A, D, or F strains (table 5)

For the isolation of the different bacteriophages, "mud" *from the L S U* sugar house was diluted in about an equal portion of water and allowed to settle for 2 days The supernatant fluid was first filtered through paper and then through a Pasteur-Chamberland filter The filtrate obtained was inoculated into a series of tubes seeded with the different strains of *L mesenteroides* Each tube containing tryptone glucose yeast extract broth was seeded with 1 loopful of one of the strains and inoculated with 1 ml of the filtrate A control tube without the filtrate was inoculated with each organism The tubes were observed after

TABLE 5

Agglutinin absorption tests with antiserum 860 (type F)

ANTIGENS		ANTISERUM 860F	
		Not absorbed	Absorbed with 956(D)
Type A	153	50	50
	732	100	50
	1,021	50	50
	1,022	50	50
	1,024	50	50
Type D	1,067	100	0
	835	200	200
	910	200	200
	956	400	0
	320	200	200
Type F	1,010	400	400
	1,011	200	200
	1,012	100	200
	860	400	400
	861	100	200
	862	400	400
	864	200	200
	866	200	200
	867	200	200
	868	50	50
	708	100	100
	711	100	200
	714	50	200

1 and 2 days for lysis, and those which showed *no growth of the organism* were reinoculated with a growing, young culture If no growth *occurred,* the bacteriophage suspension was filtered through a Pasteur-Chamberland filter and, after several repetitions of this procedure, the filtrate was used for the tests

Considerable difficulty was experienced in isolating the bacteriophages, and only 5 were obtained Each bacteriophage was tested for the formation of plaques with the homologous organism and then tested against all the strains available The bacteriophages obtained were for the strains 730 (A type), 700 and 706 (B type), and 200 and 209 (D type) No bacteriophage was obtained for any organism of the F type

The lytic activity of the bacteriophages was determined by the spot plate

surface by means of a glass rod, and the plates were again allowed to dry for 4 hours   Inoculation with phage was effected by depositing a loopful of active filtrate on a certain spot on the plate   All the bacteriophages were tested on the same plate

The results of this experiment are presented in table 6   It is apparent that each phage tested exhibited type specificity to a considerable degree   Of 105 strains only two were lysed by phages from outside the type   A high degree of strain specificity was noted, however, particularly in the D type

TABLE 6

Susceptibility of Leuconostoc mesenteroides to bacteriophages

TYPE	STRAINS TESTED	PER CENT POSITIVE REACTIONS WITH PHAGE				
		730(A)	700(B)	706(B)	200(D)	209(D)
A	18	50	0	0	5 5	0
B	31	3 2	41 9	41 9	0	0
D	42	0	0	0	11 9	9 5
F	14	0	0	0	0	0

SUMMARY

Serological and bacteriophagic studies on strains of *Leuconostoc mesenteroides* isolated from cane juice indicated that Faville's type A constitutes a reasonably distinct and homogeneous group, whereas the B, D, and F types are quite heterogeneous   Of the three tests employed the agglutination test was the most useful in showing type relationships

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▲	2 C	0 6 C	0 9 C	0 9 C
3	−0 6 C	−1 9 C	−3 2 C	—
4	0 7 C	1 5 C	1 7 C	1 7 C
5	0 5 C	1 3 C	1 4 C	1 4 C

This plasma pool affords an example of a protein solution from which the removal of pyrogenicity was effected by either of the agents alone

Five hundred ml of this plasma were filtered through an S-6 pad, 40 sq inches per liter After filtration a pyrogen test showed the following

Rabbit	1 hr	2 hr	3 hr	Max rise
1	−0 6 C	−0 7 C	−0 5 C	—
2	−0 2 C	0 4 C	0 4 C	0 4 C
3	−0 2 C	0 4 C	0 4 C	0 4 C

Five hundred ml of the pyrogenic plasma were stirred with 15 g of "decalso" for a half-hour and were filtered through an S-3 pad, 40 sq inches per liter The filtered material gave the following pyrogen test

Rabbit	1 hr	2 hr	3 hr	Max rise
1	0 2 C	0 4 C	0 5 C	0 5 C
2	−0 3 C	−0 1 C	0 1 C	0 1 C
3	−0 3 C	0 0 C	−0 1 C	—

REMOVAL OF PYROGEN FROM A 25 PER CENT NORMAL HUMAN SERUM ALBUMIN SOLUTION

A 25 per cent solution of albumin gave the pyrogen tests below Its pyrogenicity is better indicated by the fact that one of the rabbits died immediately after the test, and the other two were moribund, than by the actual temperature rises shown

Rabbit	1 hr	2 hr	3 hr	Max rise
1	0 4 C	0 2 C	0 2 C	0 4 C
2	0 4 C	0 1 C	0 1 C	0 4 C*
3	1 2 C	1 5 C	1 9 C	1 9 C

* Rabbit died

Earlier work has shown that with the highly concentrated albumin solutions the 30 g of "decalso" per liter usually used were ineffective even when followed by S-6 filtration Likewise, the use of 60 g of "decalso" per liter of solution was only slightly effective Five hundred ml of the foregoing solution were stirred one half-hour with 120 g of "decalso" (240 g per liter), and then were filtered through an S-6 pad, 40 sq inches per liter The filtered albumin gave the following pyrogen test None of the rabbits were ill

Rabbit	1 hr	2 hr	3 hr	Max rise
1	−0 7 C	−0 5 C	−0 3 C	—
2	−0 2 C	−0 4 C	−0 4 C	—
3	−0 8 C	−0 4 C	−0 3 C	—

SUMMARY

The pyrogenicity of concentrated protein solutions can often be reduced by treatment with "decalso," and by filtration through S-6 pads of the Republic series

The amount of "decalso" and the area of pad space used are functions of the degree of pyrogenicity and of the protein concentration of the solution, and for maximum effectiveness they should be determined for each solution to be examined   In practice, for 6 to 8 per cent protein solutions of mild pyrogenicity, 30 g of "decalso" and 40 sq inches of pad surface per liter of solution have given adequate reduction of pyrogenicity

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IV Comparative Responses of Gram-positive and Gram-negative Bacteria to Penicillin[1]

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It is well established that in general large differences exist in the sensitivity of gram-positive and of gram-negative organisms to penicillin, the differences are so large, in fact, that most infections due to gram-negative organisms are classed as refractory to penicillin treatment, although it is known that at sufficiently high concentrations of the antibiotic *in vitro* many such organisms are inhibited It is also well established that there are relatively wide differences in sensitivity to penicillin among different species of susceptible bacteria and even among different strains or races of a single susceptible species (Herrell, 1945) It seems of interest, therefore, to ascertain whether the same mechanism of penicillin action operates in gram-negative organisms as in gram-positive, but perhaps at a higher threshold level, or whether an entirely different mechanism of action must be sought This problem is of practical as well as theoretical importance, since, if the same mechanism is operative in both types of organisms, but merely at different threshold levels, it may be possible to find a practical means of lowering the threshold of sensitivity in the more resistant organisms and thus to bring them within the scope of effective action of penicillin in practical clinical doses The advantages to be anticipated from such a procedure are obvious, since the superiority of penicillin over other currently available antibiotics on the basis of toxicity, untoward reactions, development of fastness in the organisms under treatment, etc , is generally recognized

It is difficult, if not impossible, in the present state of knowledge to define with certainty the precise biochemical and biophysical mechanisms through which penicillin exerts its effect on susceptible organisms The fact that it exerts a strong selective action in *relatively low concentrations against many types of* bacterial cells without manifesting any appreciable toxicity toward other living structures indicates that it does not owe its action to a drastic, general protoplasmic poisoning as do many other antibacterials (halogens, salts of heavy metals, phenols, etc), but it is reminiscent rather of the "receptor" hypothesis of Ehrlich (1908, 1914), who believed that antiseptic agents act against susceptible cells by linking chemically with reactive groups contained therein Specific tests with different stains and reagents may be visualized, in a way, inasmuch as they help to reveal chemical changes that occur in cells and colonies under the

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TABLE 1

Response to different dyes and reagents on penicillin assay plates seeded with gram positive organisms

			REACTION ON ASSAY PLATES					
			Staphylococcus aureus			Bacillus subtilis		
			Color		Definition of boundary	Color		Definition of boundary
REAGENT	GROUP ASSUMED TO BE ACTIVE	AUTHORITY* AND DESCRIPTION OF TEST	Inside of zone	Outside of zone		Inside of zone	Outside of zone	
K ferricyanide, ferric sulfate	–SH	Mason, H L, 1936	Faintly bluish	Deep blue	Very sharp	Faintly bluish	Deep blue	Very sharp
Schiff's	Free aldehydes	Oster, K A, 1946	Clear, faintly pink	Red	Very sharp deep red ring of enhanced growth	Clear, faintly pink	Deep red	Sharp
Schiff's (after pretreatment of plate with HgCl2)	Aldehydes bound in cells	Oster, K A, 1946	Faintly pink	Deep red	Very sharp	Test not performed		
Osmic acid	Dienol (o poly phenols)	Dufrenoy, J, 1945	Clear	Dark	Very black ring	Clear	Dark	Sharp black ring
Azo reaction (in alkaline solution)	Dienol (o poly phenols)	Lison, L, 1936	Faintly pink	Red	Very sharp	Orange	Red	Very sharp, brilliant orange red ring of enhanced growth
Sakaguchi	Substituted guanido	Sakaguchi, S, 1925; Vincent, D, J; Brygoo, P, 1946	Clear	Pink	Very sharp	Faintly pink	Pink	Poor
Molybdate	PO_4'''	MacDougal D F, & Dufrenoy, J, 1944	Clear gray blue	Blue	Very sharp deep blue ring of enhanced growth	Golden	Blue	Sharp by transmitted light, poor by reflected light
Hematoxylin after molybdate	Lipidic complex	MacDougal, D T, & Dufrenoy J, 1944	Light blue	Deep purple	Moderately sharp Black	Faint violet	Deep violet	Poor
Toluidine blue	Ribonucleic acid	Jeener, R, & Brachet, J 1943	Lavender	Purple	Very sharp	Faintly bluish	Deep blue	Sharp
FeCl3	o Diphenols	Lemoigne M 1928	Light gray green	Greenish	Poor	Faintly bluish	Dark blue green	Sharp
Methyl green (approximately 0.6% solution)	Polynucleotides dehydroribonucleic systems	Brachet G 1942	Green	Pink	Sharp	Faintly greenish	Pink	Poor

* For complete citation see list of references at the end of the paper
† Plates in the the plate before reagents are applied

REAGENT[*]	REACTION ON ASSAY PLATES					
	Escherichia coli			Proteus vulgaris		
	Color		Definition of boundary	Color		Definition of boundary
	Inside of zone	Outside of zone		Inside of zone	Outside of zone	
K ferricyanide ferric sulfate	Faintly blue-green	Deep blue-green	Very sharp deep blue ring of enhanced growth	Bluish	Blue	Poor
Schiff's	Faintly pink	Pink	Poor	Pink	Deep red	Very sharp deep rose ring of enhanced growth
	Pink†	Red†	Sharp†			
Schiff's (after pre-treatment with HgCl₂)	Pink	Deep red	Sharp	Pink	Deep red	Extremely sharp
Osmic acid	Clear	Dark	Very sharp black ring	Clear	Dark	Very sharp black ring of enhanced growth
Azo-reaction (in alkaline solution)	Clear	Red	Sharp	Light orange	Red-orange	Sharp
Sakaguchi‡	Faintly pinkish	Pink	Poor	Faintly pinkish	Red orange	Poor
Molybdate	Blue	Deep blue	Poor§	Blue	Light blue	Good
Hematoxylin after molybdate	Blue-violet	Deep purple	Good	Bright blue violet	Deep blue (very little violet)	Sharp
Toluidine blue	Lavender	Purple	Sharp	Bluish lavender	Dark purple	Fair
FeCl₃	Light yellow green	Greenish brown	Extremely sharp	Light yellow green	Greenish brown	Good
Methyl green (approximately 0.02% solution)	Green	Pink	Sharp intense deep pink ring of enhanced growth	Green	Pink	Very sharp intense ring of enhanced growth

* See table 1 for groups assumed to react and for authorities
† Reaction on plates incubated 18 hours with no preincubation
‡ Plates must be iced before reagents are applied
§ Although definition of zones is poor when plates are viewed macroscopically especially by reflected light they are very clearly seen when plates are examined under high dry power of microscope   The cells within the zones of inhibition appear as long blue filaments and are sharply differentiated from the uninhibited cells in the normal background   Both filamentous and normal cells stand out clearly from the less intensely stained agar background

remaining subbacteriostatic, does reach a critical level that is capable of stimulating metabolism and growth   It is well known that, like other antibacterial agents, penicillin in certain subbacteriostatic concentrations may exert a stimu-

lating effect on microorganisms *in vitro* (Miller, Green, and Kitchen, 1945, Eriksen, 1946, Curran and Evans, 1947) [3]   The threshold concentration below which penicillin enhances and above which it inhibits metabolism is, however, many times greater for activity toward the gram-negative organisms than toward the gram-positive   For example, on plates seeded with *S aureus* and treated as prescribed for the FDA cylinder plate assay (Federal Register, 10, 11478-11485,
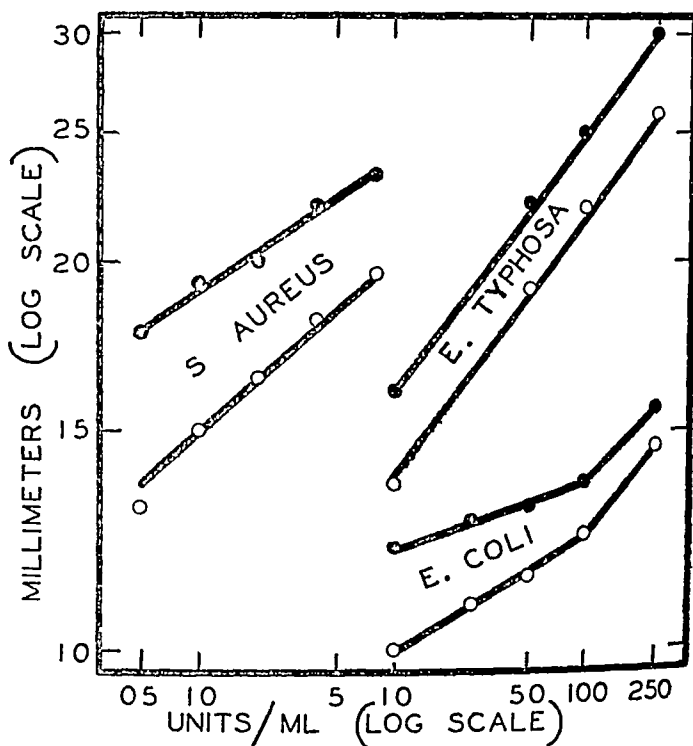
Fig 1 Diameters of Zones of Inhibition Corresponding to Different Concentrations of Penicillin on Assay Plates Seeded with S aureus, E typhosa, or E coli
    Open circles are values for plates prepared with the standard test agar   Solid circles are for same agar with COCl₂ 6H₂O added at the rate of 1 mg/L   All points are averages of values from at least 4 plates   Assays with *S aureus* were performed by the 3 hour method, others by standard overnight procedure   For convenience in plotting, all values for *E coli* have been raised 1 mm on the ordinate scale

1945), a solution containing 1 unit of penicillin per milliliter produced inhibition zones approximately 21 mm in diameter, but a solution containing approximately 100 units of penicillin per milliliter was required to produce a zone of the same diameter on plates seeded with *E typhosa* or *P vulgaris*, while solutions containing 250 units per milliliter produced zones only slightly over 13 mm in diameter on plates seeded with *E coli* (figure 1)   It is noteworthy that the addition of

[3] Evidence of similar action of penicillin *in vivo* is less convincing   The authors know of no clear-cut demonstration of such an effect in animals or human patients infected with penicillin-susceptible organisms and treated with penicillin

dosage curve is shifted along the abscissa when *E coli* or *E typhosa* is used as the test organism instead of *S aureus* and that the curve for a given organism is shifted upward on the ordinate scale when appropriate concentrations of $CoCl_2$ $6H_2O$ are added to the test agar A similar effect of cobalt in lowering the effective threshold for penicillin has been demonstrated *in vivo* (Pratt, Dufrenoy, and Strait, 1948) The enhancing action of trace amounts of cobalt on penicillin activity appears to be specific and is receiving special study in this laboratory Salts of nickel, manganese, platinum, iridium, gold, zinc, and copper have been tested in a similar manner over a wide range of concentrations and have failed to produce any similar increase in the diameters of the inhibition zones around cylinders containing penicillin

DISCUSSION

The tests that are most useful in revealing the chemical changes that occur on penicillin assay plates are those that result in clear-cut differential staining of test organisms vs agar background and of inhibition zones vs the general background of uninhibited growth, and that intensify the ring of enhanced growth These staining reactions, emphasizing the contrasts between the regions of normal growth in the background of the plates, the marginal rings of enhanced growth, and the zones of inhibition, can be interpreted from the physicochemical point of view as manifestations of differential shifts in rH and concomitant shifts in pH in the corresponding parts of the plates The various levels of rH and of pH can be estimated by the proper use of indicators From the biochemical point of view these changes may probably be ascribed in large measure to changes in the relative rates of proteogenesis and proteolysis in cells exposed to bactericidal, inhibiting, stimulating, and ineffective concentrations of penicillin Disturbance of the normal assimilative and growth metabolism might be expected to lead, in turn, to an unequal distribution of different proteins and other cellular components on different parts of the plates As shown in tables 1 and 2 these changes can be revealed by use of reagents for detecting -SH groups, aldehydes, polyphenols, guanido groups, phosphate ion, polynucleotides, fatty acids and lipids, etc Surface phenomena, due no doubt in part at least to changes in pH, cannot be ignored in interpreting these results and warrant a full discussion separately

In our experiments it was convenient to work with inhibition zones approximately 15 to 20 mm in diameter It was found in our work that to produce inhibition zones in this range on plates that were seeded with *E typhosa*, or *P vulgaris*, and to which no cobalt had been added, it was necessary to employ penicillin solutions 10 to 100 times as concentrated as when *S aureus* was used for the test organism, and that, on plates seeded with *E coli*, solutions containing as much as 250 units of penicillin per milliliter failed to produce zones of this size However, when the concentration of penicillin was adjusted so that it fell within a bacteriostatic range, the reactions for the several active groups, levels of rH and pH, etc , were as pronounced and sharp on plates seeded with gram-negative

organisms as on those seeded with gram-positive organisms, and they demon
strated a homologous pattern   In fact, plates treated with a given reagent
appeared macroscopically identical, irrespective of the test organism that was
used, if the relative times of preincubation and of secondary incubation were
chosen so that the plates were developed at the time when the sharpest differential
could be achieved

For clearest results it is essential that the interaction of the biological and the
physical factors be properly balanced   Our experience indicates that failure
of a reagent that has revealed a sharp definition of zones on plates seeded with a
given organism to "develop" properly plates seeded with another organism may
be ascribed primarily to too long a preincubation period   If the primary incu
bation period exceeds the duration of the lag period, growth of the test organisms
on the plates becomes too dense before the penicylinders[4] are placed thereon and
diffusion of penicillin is permitted to begin   Under these conditions the sub-
bacteriostatic effect corresponding to "below threshold" concentrations of peni
cillin may fail to be differentiated from the bacteriostatic effect corresponding to
"above threshold" concentrations   For example, the Sakaguchi test (tables 1
and 2) very clearly revealed the prevalence of substituted guanido groups in the
zone of enhanced growth on penicillin assay plates seeded with S $aureus$ The
failure of the reagent to provide a sharp response on plates seeded with other
test organisms may be ascribed to improper timing of preincubation or secondary
incubation periods

Methods that require flooding of the test plates with reagent solutions are sub-
ject to the criticism that the flooding operation may dislodge some of the test
organisms from their initial position on the plates and that, consequently, the
pattern which develops following the chemical treatment may fail to correspond
to the original pattern of distribution of the several reactive groups   Therefore,
to eliminate this objection, in the present experiments all results obtained with
the several reagents were checked as to sizes of inhibition zones, distribution of
enhanced growth, bacteriostasis, and bacteriolysis, on plates which, at the end
of the second incubation period, were inverted over a watch glass containing a
solution of osmic acid that was stabilized by chromic acid   It was seen that the
results on plates so treated corresponded with the observations made on plates
treated with the other reagents   The development of the plates exposed to the
vapors of osmic acid can be watched easily as it progresses—first, the ring of
enhanced growth darkens, and then it blackens as the general background dark
ens   Microscopical examination of such plates under oil immersion shows that
osmic acid is reduced in the vacuolar solution of the test organisms   The re-
duction occurs more rapidly and is more evident in the zones of enhanced growth
Plates thus "fixed" by fumes of osmic acid may subsequently be stained by ap-
propriate cytological stains, for further study of the cytochemical structure
Osmic acid is reduced rapidly to black osmium oxide in the vacuolar solution and

[4] Trade name for standard cylinders used in assaying penicillin solutions by the agar
plate method   Penicylinders are available from Eimer and Amend, New York, and from
other firms that supply laboratory apparatus

ʋ   ᵕ   ᴵ ᵕ   ɯ ᵻ ᵻ aᴄ with certain fatty ma'erials   The formation of
the black deposit in contact with phenolic compounds has never been clearly
explained in terms of physical chemistry   In view of what happens with other
metals chelation might be surmised

The results of our experiments suggest that in penicillin-sensitive organisms
the gram-positive complex, which is known to consist of a magnesium ribonu-
cleate involving a sulfhydryl group (Henry and Stacey, 1946, Bartholomew and
Umbreit, 1944), accelerates the action of the penicillin molecules in inactivating
-SH groups which form essential links in the chain of metabolic reactions in-
volved in growth   Under the effect of penicillin the "gram-positiveness" dis-
appears   This is significant in view of the hypothesis that has been developed
in this and in earlier papers (Dufrenoy and Pratt, 1947a, Pratt and Dufrenoy,
1947b), since it is known that the gram-positive complex loses its characteristics
as its -SH groups become dehydrogenated to S-S (Henry and Stacey, 1946)
In microorganisms which lack the gram-positive complex the concentration of
penicillin must be increased many times to obtain the bacteriostatic effect, but
irrespective of the minimum dosage required to produce bacteriostasis, the se-
quence of events is always the same   first the microorganisms undergo a phase of
enhanced activity, during which they develop pronounced reducing power, and
the cells at the margins beyond the range of diffusion of bacteriostatic concentra-
tions of penicillin manifest the characteristic symptoms of the logarithmic phase
of growth   This is the period during which the dehydrogenases are most active,
and the rH of the medium tends to drop to the lowest value   This change is
evidenced by reduction of Redox indicators   During this phase of growth the
organisms store phenolic compounds in their vacuolar solutions which, therefore,
acquire the aptitude to absorb (or adsorb) basic fuchsin, phenosafranine, neutral
red, etc   The phenolic compounds can be demonstrated by the action of mild
oxidants such as potassium dichromate or potassium iodate, which oxidize them
to brownish yellow quinoid derivatives   Conversely they can also be demon-
strated in the vacuolar solution of the bacteria by virtue of their reducing action
toward osmic acid, silver nitrate, etc , or by the formation of darkly colored
metallic complexes with ammonium molybdate (Marchal and Girard, 1947) or
with ferrous salts   Where positive reactions can be obtained for phenolic com-
pounds, a sharp positive reaction can be obtained for -SH groups through the
formation of Prussian blue, by treatment with potassium ferricyanide followed by
ferric sulfate

One other point should be mentioned in connection with the observation that
a ring of enhanced growth always surrounds the zones of inhibition on penicillin
assay plates   It has been pointed out above that these rings of enhanced growth
probably represent a visible manifestation of enhanced metabolism induced in
cells in that region by subbacteriostatic concentrations of penicillin   It should
be observed, however, that other factors may contribute to the enhancement of
growth in those areas circumjacent to the areas where growth is inhibited and
lysis of cells occurs   It is not impossible that as cells in the areas of inhibition

are affected by bacteriostatic concentrations of penicillin, some of their compon
ents are liberated into the agar, through which they may diffuse to regions of the
plate in which the concentration of penicillin fails to reach a bacteriostatic level
A number of references in the literature indicate that products liberated by dying
microorganisms may serve as growth factors for survivors (Nicolle and Faguet,1947,
Lasfargues and Delaunay, 1947, Cook and Cronin, 1941, Loofbourow, 1947, Webb
and Loofbourow, 1947)   Some of these substances, especially the nucleopro-
teins, might then be absorbed by the bacterial cells outside of the zones of
inhibition and serve as metabolites or growth factors   Support for such a
hypothesis is afforded by the experiments of Bonét-Maury and Perault (1945)
By the use of a recording photometer they observed that when S aureus was
cultured in the presence of small amounts of penicillin in broth two waves of growth
occurred   The results may be interpreted as indicating that when S aureus
cells are suspended in broth containing very small amounts of penicillin, the
most sensitive organisms, which are first affected, release into the medium sub-
stances that promote a second wave of growth among the more resistant cells
Therefore, the possibility should not be overlooked that the enhancing action
that is apparent as a "space" effect on assay plates is comparable in some meas-
ure to the action which the recording photometer demonstrates as a "time"
effect in suitable broth cultures

SUMMARY

A study has been made of physical and chemical changes that occur in different
parts of penicillin assay plates seeded with gram-positive and with gram negative
test organisms   The techniques that were used were intended to reveal differ
ential changes that occur in cells under the influence of bactericidal, lytic, in
hibiting, and stimulating concentrations of penicillin as contrasted with the re-
action of cells in the normal background where the concentration of penicillin
remains ineffective

It was found that the same pattern developed on all penicillin plates treated
with a given reagent, irrespective of the test organism, provided the proper
concentrations of penicillin were used and provided the proper balance of the
biological and physical factors involved in the cylinder plate method of assay
was achieved   The latter was found to be largely a matter of properly controlling
the relative lengths of the primary incubation period, when the organisms were
in the lag period, and of the secondary incubation period, during which the
organisms were in the log phase of growth and during which penicillin was dif
fusing through the medium in the plates

The evidence indicates that penicillin affects aerobic gram-positive and gram
negative organisms through the same chemical systems   The threshold concen
tration at which its effects become manifest is, however, many times greater on
plates seeded with gram-negative organisms than on those seeded with gram
positive organisms

The proper use of trace amounts of cobalt lowers the effective threshold on
test plates, a fact which may have practical clinical importance as well as theo-
retical interest, since the same phenomenon has been demonstrated in vitro

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ᴀ... ᴜ ᴊ ... ᴛ ... ᴊ ᴋ   CTS OF X-RAYS ON ESCHERICHIA COLI AS RELATED TO THE YIELD OF BIOCHEMICAL MUTANTS

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The isolation of growth-factor-requiring mutant strains of *Escherichia coli* from cultures exposed to X-rays during growth in a broth medium, and in one case from a culture transferred serially in broth without X-ray treatment, has been reported (Roepke, Libby, and Small, 1944)   The yield of mutant strains was relatively low and variable, and the procedures would tend to limit the mutant strains to those able to grow at least as rapidly as the parent strain   Gray and Tatum (1944) and Tatum (1945) have reported the isolation of similar mutants from cultures of *E coli* exposed for a short time to X-rays of high intensity The X-rayed cultures were incubated 4 hours in a broth medium before plating for single-colony isolation   This procedure, although giving a relatively high yield of biochemical mutants, also tends to favor the isolation of those strains with the highest rate of growth

This report deals with attempts to obtain biochemical mutants of *E coli* by a procedure not affected by their relative growth rates and to determine some of the factors affecting the yield of mutant strains

Theoretical considerations   From quantitative studies of the lethal effects of X-rays on *E coli*, Wyckoff (1930) and Lea et al (1936, 1941) concluded that death of a bacterial cell was the result of a single "hit" or absorption of a single quantum of X-ray energy in a vital structure in the cell   Lea and his coworkers (1941) suggested that these vital structures may correspond to genes and that death of a bacterial cell by X-rays may be considered as a lethal mutation If this assumption is valid, the number of viable mutations produced by X-rays should increase in direct proportion to the number of cells killed by X-rays On the basis of this assumption and with the further assumption that a viable mutation does not alter the sensitivity of the cell to X-rays, one may derive a relationship between the ratio of viable mutant to nonmutant cells and the survival ratio

According to the "one-hit-to-kill" theory the log of the survival ratio decreases linearly with the time of X-ray treatment (Wyckoff, 1930)   Thus,

$$\ln \frac{N}{N_0} = -kt \quad \text{or} \tag{1}$$

$$\frac{dN}{dt} = -lN, \tag{2}$$

where N_0 = the initial number of viable cells, N = the number of surviving cells at any time, t, and k = a constant dependent on the intensity of the X-rays

If x = the number of viable mutant cells of a particular type, i e , cells unable to carry out reaction X, n = the number of all other viable cells, n_0 = the initial number of such cells, and R = the mean ratio of cells mutated (to mutant X) to cells killed by X-rays, then the rate at which mutant cells of type X are killed = kx, the rate at which all other cells are killed = kn, and the rate at which mutant cells are produced = Rkn, from which

(3) $$\frac{dx}{dt} = -kx + Rkn \quad \text{and}$$

(4) $$\frac{dn}{dt} = -kn - Rkn$$

Substituting from $\dfrac{dx}{dt} = \dfrac{dx}{dn} \dfrac{dn}{dt}$, equations 3 and 4 may be combined and integrated to give

(5) $$\frac{x}{n} = \frac{K}{n^{(R/(1+R))}} - 1,$$

where K = the constant of integration

If $\dfrac{x}{n}$ = r when $n = n_0$, then $K = (1 + r)\ n_0^{(R/(1+R))}$ and substitution in equation 5 gives

(6) $$\frac{x}{n} = (1 + r) \left(\frac{n_0}{n}\right)^{(R/(R+1))} - 1$$

According to this relationship the ratio of mutant cells of a specific type to all other cells surviving X-ray treatment varies in an inverse manner with the survival ratio   From the derivative of $\dfrac{x}{n}$ with respect to n, it can be shown that $\dfrac{x}{n}$ continues to increase without reaching a finite maximum as the survival ratio approaches zero   In other words, the greater the percentage of cells killed by X-rays, the greater will be the percentage of mutant cells among the survivor If $R > r$, the value of x will pass through a maximum as the survival ratio decreases   However, in the case of biochemical mutants which cannot be isolated by a selective method one is interested in obtaining a high percentage of mutant cells in order to facilitate their isolation

Equation 6 is dependent on the validity of the assumptions stated previously and on the use of experimental conditions that insure uniform exposure of the cells to X-rays   Since R in this equation is a ratio of probabilities with a variation around a mean value, the ratio $\dfrac{x}{n}$ may be expected to show considerable experimental variation as the number of surviving cells approaches zero   Thus

value of R Up to the range in which the number of double mutations becomes significant, x may be considered as the number of mutant cells of a general type, i e , growth-factor-requiring cells

Although equation 6 may be valid over an appreciable range of X-ray dosage, the experimental results indicate that it does not hold for more extensive X-ray treatment, the limitations being dependent to some extent on the experimental conditions

METHODS

$E\ coli$ no 15[1] was used in this study   Preparatory to X-ray treatment, single colony cultures were inoculated into the basal synthetic medium consisting of inorganic salts, glucose, and asparagine (Roepke et al , 1944)   In experiments involving "young" cells cultures in the basal medium were started with sufficiently large inocula to give visible turbidity and incubated until approximately half the maximum turbidity was obtained   The cultures were then cooled to 15 to 20 C to reduce the growth rate during centrifugation   "Old" cells were harvested from cultures started with a small inocula and incubated for 21 to 28 hours at 37 C, maximum growth being obtained within 18 hours   The harvested cells were washed once and resuspended in saline-phosphate buffer (0 50 per cent $NaCl + 0\ 20$ per cent KH_2PO_4 adjusted to pH 7 0 to 7 2 with NaOH) to give viable counts of about 1×10^9 cells per ml in the first two experiments and 1×10^9 to 7×10^{10} cells per ml in subsequent experiments   Unless stated otherwise, X-ray treatment was initiated within 2 hours after preparation of the cell suspension

In preliminary experiments the cell suspensions were X-rayed in the aluminum chamber used previously (Roepke et al , 1944)   The X-ray beam was admitted through a waterproofed cellophane window, and the suspension was stirred with a motor-driven glass stirrer   When it became evident that stirring alone was not sufficient to attain uniform exposure of the cells, the aluminum chamber was replaced with a pyrex test tube, 15 by 150 mm, the lower end of which was blown into a bulb 22 mm in diameter   The tube, containing 1 6 ml of cell suspension, was placed about 75 mm from the window of the X-ray tube and in such a position that the cross section of the X-ray beam more than covered the cross section of the cell suspension, including the walls of the pyrex tube several mm above its junction with the suspension   The suspension was stirred and the pyrex tube rotated continuously during X-ray treatment   The temperature of the suspension during incubation was maintained at 19 to 20 C in the first few experiments and at 10 to 14 C in the later experiments

X-ray treatment was carried out with a General Electric X-ray diffraction unit, using a tube with a molybdenum target at 40 kv and 19 to 20 ma

In the determination of the death curve samples of approximately 0 05 ml were removed with micropipettes at intervals of 15 to 45 minutes   The X-ray

[1] No 9,723 of the American Type Culture Collection

treatment was interrupted for 3 to 5 minutes during removal of the samples
The ability of a cell to grow into a visible colony on agar medium was used as the
criterion of viability   Viable counts were made by plating with Difco nutrient
agar and incubating 24 to 30 hours at 37 C

The general procedure used in the isolation and identification of biochemical
mutants has been described (Roepke *et al*, 1944)   Difco AC broth supple-
mented with a crude liver extract was used as the "complete" medium and will
be referred to as AC broth   In some cases this was supplemented also with an
autolyzate prepared from baker's yeast and sterilized by filtration   For single-
colony isolation various dilutions of the X-rayed suspensions were flooded on the
surface of AC agar (AC broth plus 2 per cent agar), and the excess was drained
to one edge of the plate and removed with a pipette

RESULTS AND DISCUSSION

In previous studies (Roepke *et al*, 1944) in which cell suspensions contained
in the aluminum chamber were exposed to X-rays, it was found that the decrease
in the log of the survival ratio was essentially linear with time, although irradia
tion treatment was not extended beyond that giving a survival ratio of about
10^{-3} With more extensive X-ray treatment the plot of the log of the survival
ratio versus the time of exposure deviated appreciably from linearity at low
survival ratios (curve A, figure 1)   In this experiment the samples were re-
moved from the container without additional mixing   When the cell suspension
was mixed with a pipette just prior to removal of the samples, the death curve
was very irregular at low survival ratios, indicating that the motor-driven stirrer
was not sufficient to insure uniform exposure of the cells

The use of the pyrex tube in place of the aluminum chamber insured relatively
uniform exposure of the cell suspension (curves B and C, figure 1)   Some
deviation from linearity is evident, but this is always in the direction of an
increased death rate with time of X-ray treatment   The deviations from line-
arity in the first part of the curves obtained with suspensions of young cells
(figures 1 and 2) are probably due to a discrepancy between plate counts and
the number of viable cells   The cells harvested from actively growing cultures
may occur in large part as short chains of incompletely divided cells (Robinow,
1945), so that many of the colonies obtained in plate counts may be derived from
two or more viable cells   Thus, in the control suspension and in the early period
of the X-ray treatment the actual number of viable cells would be higher than
that indicated by plate counts, with the two values approaching equality as more
cells are killed

Curve D, figure 1, is a theoretical curve showing the relation between the log
of the survival ratio, on the basis of plate counts, and the time of X-ray treat-
ment when all of the cells exist in chains of four cells each with all of the cells
initially viable   The calculations are based on the assumption that the death
rate of individual cells follows equation 1 [2]   As shown by the calculated curve E,

[2] On the basis of equation 1 the death of cells occurring in chains of 4 may be considered
as analogous to a series of 4 consecutive, first order reactions, as

ratios plate counts can be considered as an adequate indication of the number of viable cells   From the point of intersection of the linear portion of the experimental curves with the zero axis, it appears that the number of viable cells
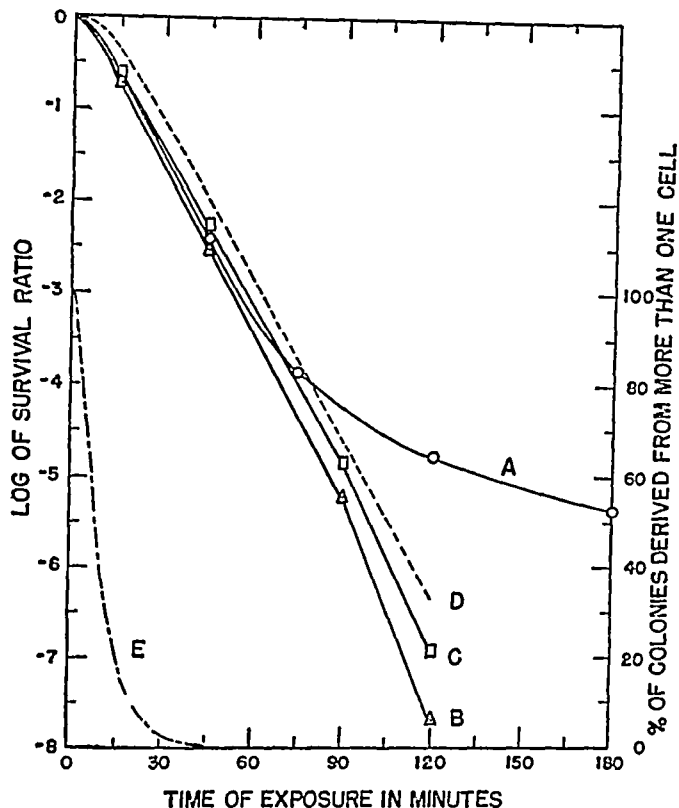
FIG 1   X-Ray Treatment of Young Cells

A, suspension X-rayed in aluminum chamber, *B* and *C*, 2 portions of same suspension in pyrex tube, X-rayed immediately after preparation (*B*) and after storage at 4 C for 20 hours (*C*), *D* and *E*, theoretical curves of log of survival ratio as indicated by plate counts (*D*) and percentage of colonies originating from more than one viable cell (*E*), calculated on the supposition that all of the cells occur in chains of 4 cells each (see text)

$$ a \xrightarrow{\ k_1\ } b \xrightarrow{\ k_3\ } c \xrightarrow{\ k_3\ } d \xrightarrow{\ k_4\ } e $$
$$ 0000 \qquad X000 \qquad XX00 \qquad XXX0 \qquad XXXX $$

where a = the number of chains in which all 4 cells are viable, b = the number of chains in which one cell has been killed, etc   Since the probability of killing any one of the viable cells in a chain is directly proportional to the number of viable cells in that chain, the rate constant $k_1 = \frac{4}{3}k_2 = 2k_3 = 4k_4$ The value of k_4 (8/hour) was approximated from the relatively linear portion of one of the experimental, death rate curves   The viable count as indicated by plate counts corresponds to the sum $a + b + c + d$ The values of $a, b, c,$ and d were calculated by means of the general equations used in calculating the variation with time of the quantity of decay products in a radioactive series (Rutherford *et al* , 1930)

initially present in most of the suspensions of young cells was about twice that indicated by plate counts   This is in general agreement with the observations of Robinow (1945) on the structure of rod-shaped bacteria   In a few experiments the shape of the death rate curve indicated an appreciably greater discrepancy between plate counts and the number of viable cells (curve A, figure 2) This may have been the result of incomplete dispersion of the centrifuged cells
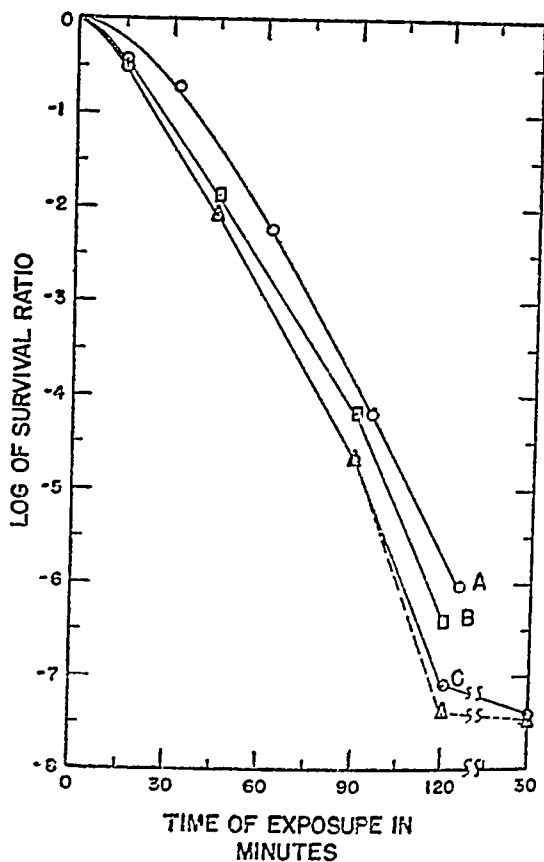
FIG 2 X-Ray Treatment of Young Cells

A and B, cell suspensions X-rayed immediately after preparation, C, portion of same suspension as B X-rayed after storage at 4 C for 24 hours, ——, plates incubated at 37 C, and - - - - at 25 C   The last points on curves C obtained by keeping X rayed suspension at 14 C for an additional 30 minutes before diluting and plating

In two experiments the viable counts on the control (nonirradiated) suspensions increased 20 and 25 per cent during incubation at 20 C for 5 and 3 hours, respec tively   However, this degree of growth or increased dispersion of the cells would not appreciably affect the shape of the death rate curve

Death rate curves were determined on only two suspensions of old cells (figure 3)   With one suspension, on which two runs were made after storage at 4 C for 18 and 22 hours, the change in slope in the initial portion of the curves is such as to indicate a heterogeneity of the cells with respect to X-ray sensitivity

mutation affecting X-ray sensitivity in the culture from which the cells were harvested. However, storage of suspensions of young cells before irradiation did not result in a change in shape of the death rate curves (figures 1 and 2). A mutation resulting in increased resistance to ultraviolet and X-ray irradiation has been reported by Witkin (1946).
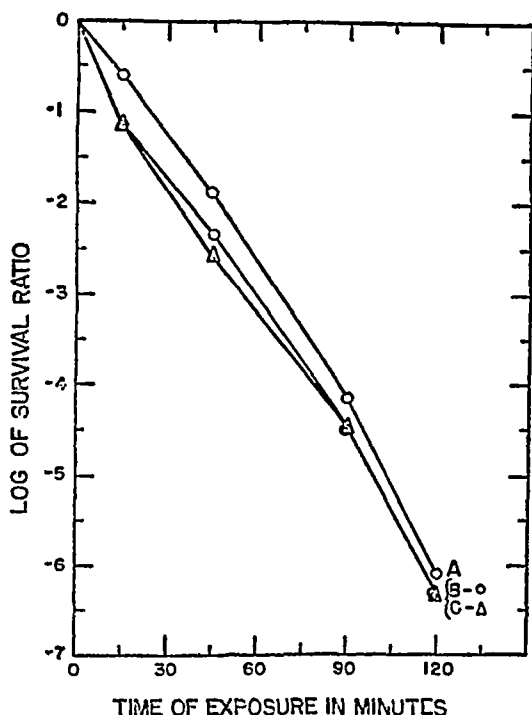
FIG 3 X-Ray Treatment of Old Cells

A, cells harvested from 28-hour culture and X-rayed immediately, *B* and *C* cells harvested from 21-hour culture and X-rayed after storage at 4 C for 18 (*B*) and 22 hours (*C*)

The deviation of the death rate curve from linearity at low survival ratios (figures 1, 2, and 3) is probably due, in major part at least, to the cumulative effects of nonlethal hits in the less vital structures of the cells, resulting in increased fragility, or decreased viability, and finally death of the cells. This is indicated by the observations that the viable count of suspensions in the region of low survival ratios (10^{-6} or less) is dependent on the nature of the plating medium, on the interval between cessation of X-ray treatment and the time of plating, and on the temperature of incubation of the plates. Similar effects were observed by Hollaender (1943) following ultraviolet treatment of *E coli* cells. It was found that the viable count of cells surviving ultraviolet treatment decreased during incubation in a phosphate buffer at a rate dependent on the

extent of ultraviolet treatment and the temperature of incubation   The sur
viving cells also showed a prolonged lag phase when incubated in broth

Plate counts on X-rayed suspensions with survival ratios in the range of 10^{-4}
or less were found to be significantly lower (one-half to one-tenth) with AC agar
than with Difco nutrient agar, and even lower counts were obtained with trypti
case soy agar (Baltimore Biological Laboratories)   On suspensions with higher
survival ratios (10^{-5} or higher) the three media gave identical plate counts,
within experimental error, although Difco nutrient agar gives appreciably slower
growth and smaller colony size than the other two media   Mineral analyses of
the three media (table 1) suggest an increased sensitivity of the X-rayed cells to

TABLE 1

The mineral composition of broth media

	MILLIMOLES PER LITER					
	Na	K	Ca	Mg	Cl	PO₄
Difco nutrient broth	8 25	5 37	0 0	0 46	2 34	2 95
AC broth	50 4	13 8	0 79	0 93	23 0	6 46
Trypticase soy broth	127	30 9				

TABLE 2

*Effect of electrolyte concentration of plating medium on the viable
count of a cell suspension X-rayed for 110 minutes*

AGAR MEDIUM	CONCENTRATION OF Na PLUS K	VIABLE COUNT*
	mM per liter	
Difco nutrient	13 6	46,400
80% AC†	51	23,300
AC	64	15,400
AC + KCl	101	5,320
AC + NaCl	152	1,620

* Colony forming organisms per ml   The viable count on the nonirradiated suspension
was $3\ 03 \times 10^{10}$ cells per ml

† AC agar diluted with sterile water

salt concentration, since the viable count varied in an inverse manner with the
concentration of electrolytes in the media   This is further indicated by the
results of an experiment given in table 2   The addition of NaCl or KCl to AC
agar resulted in a lower plate count on the X-rayed suspension, whereas dilution
of the medium resulted in a higher count

In one experiment the cells were washed and resuspended in dilute (0 005 M)
phosphate buffer in place of the saline-phosphate buffer (0 086 M NaCl plus
0 015 M phosphate)   Although the death curve was not determined, the survival
ratio after 2 hours of X-ray treatment was 10^{-9} or less (no colony-forming cell in
0 10 ml with $2\ 2 \times 10^{10}$ viable cells per ml in the control suspension)   Since
similar treatment of cells suspended in the saline-phosphate buffer gave survival

very ɔw v ɪ   An increased sensitivity of the cells to electrolyte concentration may be due to increased permeability of the cells as a result of X-ray treatment This is suggested by the studies of Ting and Zirkle (1940), who found that extensive X-ray treatment of blood produces a marked increase in permeability of the erythrocytes to potassium and sodium, resulting in swelling and finally lysis of the cells

In two experiments in which suspensions of young cells were X-rayed for 120 and 140 minutes, several large clumps were noticed in the suspensions at the end of the treatment   In a third experiment in which a suspension was X-rayed for 125 minutes, the clumps appeared after incubation of the suspension in AC broth, with stirring, for 60 minutes   One or two large clumps appeared in each case with a large proportion of the cells remaining in suspension   The clumps were gelatinous in nature and difficult to disperse   Examination of stained films of such clumps showed a number of cells enmeshed in a homogeneously stained material, indicating that some of the cells had been lysed

In the remaining experiments (X-ray treatment of 110 to 125 minutes) no clumping or agglutination was evident even when the X-rayed suspensions were stored for periods of 1 hour to several days   Thus, the decrease in viable counts observed during storage of the X-rayed suspension does not appear to be due, entirely at least, to an increased clumping of the cells   This is indicated also by the effect of the temperature of incubation of the plates on the viable count of X-rayed suspensions (curves. C, figure 2)

Under our experimental conditions, then, the results indicate that down to a survival ratio of about 10^{-5} the log of the number of viable cells decreases linearly with the time of X-ray treatment in accordance with the "one-hit-to-kill" theory of the bactericidal effect of X-rays   With more extensive treatment an increasingly greater proportion of the cells dies from the cumulative effects of "nonlethal" hits   In the latter range of X-ray dosage the ratio of cells mutated to cells killed would probably decrease, in which case equation 6 would not be valid   If a viable mutation does not alter the sensitivity of the cell to such cumulative effects, the yield of mutant cells would continue to increase with X-ray dosage but at a relatively slower rate   The data given in table 3, however, show that this may not be the case   The results of experiments 460 and 482 indicate that the mutant cells die at a greater rate than do the nonmutant cells during storage of the X-rayed suspensions   Cultures of the isolated mutant strains do not appear to differ significantly from the nonirradiated parent strain as regards viability or sensitivity to environmental factors, although this aspect has not been investigated in detail

These results indicate that the ratio of mutant to nonmutant cells in the surviving cells may pass through a maximum in the range in which an increasing percentage of the cells dies from the cumulative effects of "nonlethal" hits The yield of mutant cells in this range of X-ray dosage may be increased by the use of experimental conditions that permit a greater proportion of the injured

cells to grow into colonies on the complete medium   This may be accomplished
by the use of more suitable suspending and plating media, by plating as soon as
possible after cessation of X-ray treatment, and by irradiating with a higher
intensity of X-rays   The latter would permit irradiation with a given dosage
in a shorter time and hence would reduce the incidence of death of injured cells
before the suspension is plated for single-colony isolation

With X-ray treatment of *Neurospora*, Sansome, Demerec, and Hollaender
(1945) found the percentage of mutants to increase with dosage without reaching
a maximum over the range studied (to a survival ratio of 10^{-4})   With ultraviolet
irradiation of fungi the percentage of mutant cells was found by Hollaender and
Emmons (1941) and Hollaender *et al* (1945) to pass through a maximum at a
survival ratio of about 10^{-2} In contrast to our results with X-ray irradiation of
bacteria, however, Hollaender and Emmons (1941) observed that with more
extensive ultraviolet treatment the survival ratio and the yield of mutant cells
were increased when the irradiated spores were incubated in a salt solution before
plating

Demerec (1946), in a study of X-ray-induced mutations to virus resistance in a
strain of *E coli*, found that the yield of virus-resistant cells or colonies was in
creased appreciably when plates of the irradiated cells were incubated for several
hours before applying the virus as a selective test for the mutant character
From this one might expect an increase in the yield of growth-factor-requiring
mutants if the irradiated cells were incubated for a time in a broth medium before
plating for single-colony isolation   This would not be true, however, if the
results obtained by Demerec were due only to a delay in the manifestation of the
mutation with all of the descendants exhibiting the mutant character   This has
been suggested as a possibility by Demerec and appears to be substantiated by
the results of experiment 472 (table 3), in which it was found that incubation of
the X-rayed cells in AC broth before plating failed to increase the percentage of
biochemical mutants   The data, however, are not sufficient to warrant a
definite conclusion

Although the data given in table 3 serve to illustrate the yield of biochemical
mutants that can be obtained with X-ray irradiation, they are inadequate
to demonstrate a relation between the yield and survival ratio since all of the
variables were not adequately controlled   The time of plating varied from
about 15 to 45 minutes after cessation of X-ray treatment, and the plates were
incubated at temperatures varying from 20 to 37 C   In experiments 495 and
546 the X-ray intensity, as indicated by the death rate, was about half that used
in other experiments   In experiment 468 the cell suspension appeared to be
heterogeneous as regards sensitivity to X-ray radiation (curve B, figure 3)
Relatively few colonies were available for isolation in some experiments owing to
failure to pour a sufficient number of plates with the proper dilution of the
X-rayed suspension

The classification of mutant cultures (table 3) is somewhat arbitrary   Mo-
of those classified as unstable or questionable are so unstable as to make identifi
cation of the growth requirements difficult or uncertain, whereas others appear

~~ted as stable mutants are relatively unsta~~, although ~~the~~ growth factor requirements could be readily determined   The number of different mutant strains can be considered only as the minimum number, since the mutations can be differentiated only on the basis of known differences in the growth requirements   Although identical mutant strains may have been isolated from the

TABLE 3

The yield of biochemical mutants obtained by X-ray irradiation of suspensions of E coli

EXPERIMENT NO	AGE OF CULTURE	SURVIVAL RATIO	NUMBER OF COLONIES ISOLATED		NUMBER OF MUTANT CULTURES		
					Stable		Unstable or questionable
			Plated immediately*	Plated after storage	Total	Different strains	
450	young	$1/3 \ 7 \times 10^5$	16	—	1	1	0
460	young	$1/1 \ 4 \times 10^5$	1,090	—	14	10	2
		$1/1 \ 4 \times 10^5$	—	911†	0	0	1
462	young	$1/8 \ 7 \times 10^4$	1,023	—	20	13	3
482	young	$1/1 \ 3 \times 10^6$	329	—	8	8	1
		$1/1 \ 3 \times 10^5$	—	125‡	0	0	0
489	young	$1/6 \ 5 \times 10^5$	926	—	13	11	1
495	young	$1/1 \ 5 \times 10^4$	300	—	0	0	0
546	young	$1/2 \ 7 \times 10^5$	311	—	1	1	0
468	old	$1/2 \ 1 \times 10^5$	232	—	1	1	0
		$1/2 \ 1 \times 10^5$	—	55§	0	0	0
472	old	$1/2 \ 2 \times 10^5$	188	—	3	3	0
		$1/2 \ 2 \times 10^5$	—	100‖	0	0	0
Totals	young		4,000	—	57	28	11
			—	1,036	0	0	1
	old		420	—	4	4	0
			—	55	0	0	0

* Irradiated suspension plated within 45 minutes after cessation of X-ray treatment
† Suspension diluted in saline buffer and stored at 4 C for 1 to 8 days
‡ Suspension diluted in AC broth and stored at 4 C for 15 hours
§ Suspension diluted in saline buffer and stored at 4 C for 24 hours
‖ Suspension diluted in AC broth and incubated at 37 C for 3 hours, resulting in a reduction in the viable count of 71 per cent

same irradiated suspension, it is likely that such mutations arose independently, since there was little chance for a mutated cell to divide before the suspension was plated on the agar medium   One of the mutant strains isolated from irradiated suspensions of old cells differed from any of those obtained from young cells

Only 10 contaminant colonies were obtained from a total of 5,611 isolated colonies   Eight of these were obtained in one experiment (no 482) and were identical in morphology   A colony was considered as a contaminant if the cells

differed appreciably from E $coli$ both in morphology and growth requirements
None of the strains considered to be mutant differed noticeably in morphology
from E $coli$ with the possible exception of a strain requiring thymine   The
cells of this strain were considerably elongated or occurred in long chains when
grown in limiting concentrations of thymine nucleotide   The characteristics
and growth requirements of the mutants obtained in these experiments will be
described in subsequent reports

SUMMARY

Suspensions of *Escherichia coli* were irradiated with X-rays under conditions
which insured essentially uniform exposure of the cells   The log of the survival
ratio was found to decrease linearly with the time of irradiation, in accordance
with the "one-hit-to-kill" theory of the bactericidal effects of X-rays, down to a
survival ratio of about 10^{-5} A deviation from linearity in the initial portion of
the death rate curve is considered to be the result of a discrepancy between plate
count and the number of viable cells

With more extensive X-ray treatment the death rate increases, apparently as
the result of the cumulative effects of "nonlethal" hits, which render the cells
more fragile or more sensitive to environmental conditions   Viable mutations
appear to result in an increased sensitivity to such cumulative effects as indicated
by the effect of storage of the X-rayed suspensions on the yield of biochemical
mutants

A total of 61 growth-factor-requiring mutant cultures, consisting of at least 29
different strains, were obtained from 4,420 colonies isolated from plates poured
shortly after X-ray treatment of resting cell suspensions   No mutants were
obtained from 1,091 colonies isolated from plates poured after storage of the
X-rayed suspensions at 4 C for 15 hours to 8 days

The results of this study illustrate some of the factors to be considered in a
quantitative study of X-ray-induced mutations or in an attempt to obtain high
yields of biochemical mutants

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populations of *P stewartu* in different environments, both from the standpoint of pure cultures of a single type and of known mixtures of cultures of differing types

EXPERIMENTAL MATERIALS AND METHODS

Two cultures of *P stewartu* and variants of each stock were used in these experiments stock 400 produces a small, compact, dry, highly colored colony on nutrient glucose agar, stock 500 produces a large-spreading, mucoid, nonpig mented colony   All stocks used were plated repeatedly (5 to 7 times) from isolated single colonies   With this species McNew (1938) has shown that a high percentage of the colony loci on poured agar plates are seeded with single cells After 3 or more platings the probability is tremendously high that at least once in this process the colony picked originated from a single cell

To observe the rate of mutation bacteria from a single colony were seeded into test broth and allowed to grow for the desired time   With nutrient broth at 24 C this period was 24 hours, during which approximately 16 generations oc curred   After growth the broth culture was vigorously and repeatedly shaken over a period of half an hour to obtain as many bacteria as possible occurring as individual cells   A dilution was then made so that one drop of liquid would contain the approximate number of bacteria desired per plate   In all cases it was desired to obtain mature colonies, individual and distinct from one another, so that colony characteristics could be easily observed   One drop of broth of the desired dilution was placed on the surface of a hardened agar plate and smeared evenly over the entire surface of the plate by means of an **L**-shaped rod   Plates were incubated at room temperatures for 48 hours, then observed at 9X magnification for colony classification   Reflected light at 75 degrees from the lens was used   The mutation rate was calculated by formula 2 of Demerec and Fano (1945)

$$r = 3\ 32\ aN \log_{10} \frac{CaN}{0\ 693}$$

in which

r = likely average number of mutant bacteria per culture,
C = number of cultures,
N = average number of bacteria in C cultures,
a = the mutation rate per division cycle
Their published constant of 1 6 is here corrected to 3 32   It is apparent from the values published in table 3 of their paper that they must also have used the constant 3 32   This formula is a modification of equation 8 of Luria and Del brück (1943), and these authors have discussed the assumptions on which the derivation of this equation is based   Incorrectness of any of these assumptions tends to make the calculated mutation rate greater than the real rate   It is probable that the actual mutation rate is greater than the calculated rate since factors such as mutant cell lethality, lowered viability, slower generation times, possible chromatin segregation mechanism, inability to observe small changes and random loss of variants by using small samples from a population all tend to keep the number of mutations observed at a minimum

.. .. .ges   to selection   With the mixed cultures technique only variants widely different from each other as regards colony characteristics were used

EFFECT OF TEMPERATURE UPON THE RATE OF MUTATION

Growth of *P steuartu* occurs in nutrient broth between the temperatures of approximately 10 and 38 C   Five temperatures within this range were selected to determine how the rate of mutation is affected by growth temperature   These temperatures were 12, 18, 24, 30, and 36 C

Bacteria of a single colony were suspended in nutrient broth, and after repeated shaking five identical cultures were made in which the test broth contained approximately 3,000 bacteria per ml   At this time platings were made to determine variation initially present   One tube was then incubated at each of the five test temperatures   Since bacterial growth occurred at different rates at the different temperatures, platings were made to estimate both the number of bacteria per ml of broth and the variation present after approximately the same amount of growth had occurred in each culture as indicated by faint clouding of the broth cultures   The amount and rate of mutation occurring in these experiments has been summarized in table 1

The observed variation in the original inoculum was zero   This low variability was obtained, after preliminary experiments, by maintaining stocks at low temperatures and by picking a colony to use as the initial inoculum from an agar plate containing only typical colonies   As shown in table 1, the mutation rate increases as growth temperature increases, both as regards the number and the kinds of mutant colonies observed   In the average tube at 12 C variability was rare, however, several different variants, often in large numbers, were observed in the average tube at 36 C   The fact that a sample from each colony population was grown at each of the five temperatures makes the observed trend for mutation to increase as temperature increases very significant

Stock 401 originated as a mutant of stock 400 and appears identical with the parent in all respects except for the change from dark yellow to pale yellow colony color   The mutation rate of the pale yellow stock is lower at all temperatures than the mutation rate of the dark yellow stocks, and this difference is highly significant when one stock is compared with the other at all temperatures It is apparent that some change in the genic balance has occurred because of the mutation of the dark yellow locus to pale yellow, resulting in greater genetic stability   It is probable that each mutation affects the genic balance and usually has pleiotropic effects on the organism

The differential effect of temperature on mutation of specific characters is particularly striking   The rate of mutation from dark yellow to pale yellow is changed significantly between the temperatures of 24 and 30 C, being relatively stable at the temperatures of 24 or less and becoming increasingly mutable at 30 and 36 C   The changes from dark yellow to white or from pale yellow to white also are increased at the temperatures of 30 to 36 C   All these changes are from a darker color to one of less intense color   The change from pale to dark

does the mutation from dark yellow to white   These observations are probably related and would be expected if the factors for dark yellow, pale yellow, and white colony color were part of a multiple allelic series of genes   The occurrence of small colony type in the pale yellow stock also is higher than in the dark yellow stock

Considering the mutation rate at 12 C as the control, the temperature coefficient (Q_{10}) for mutation in the dark yellow stock is approximately 5 0, whereas in the pale yellow stock the coefficient is about 2 5   Plough (1941) observed that mutation frequency in the first and second chromosome of *Drosophila* had a temperature coefficient in the neighborhood of 5 0, whereas Muller (1928) observed a Q_{10} between 2 and 3 for mutation in *Drosophila*

TABLE 3

Percentage of dark yellow type colonies when an unstable dark yellow stock segregating for dark yellow and pale yellow colony type was grown at three temperatures

		DARK YELLOW COLONIES								
Colony	Initial	33 C			24 C			15 C		
		24 hr	72 hr	96 hr	24 hr	72 hr	96 hr	24 hr	72 hr	96 hr
	%	%	%	%	%	%	%	%	%	%
1	40	52	31	11	70	62	60	83	80	82
2	38	31	17	6	34	44	42	35	65	58
3	48	31	24	16	36	37	40	62	60	61
4	54	32	20	21	57	50	46	56	67	72
5	43	28	22	14	47	51	50	53	69	64
6	25	25	15	11	37	40	38	44	49	51
7	73	31	20	7	58	62	58	67	74	70
8	70	27	22	15	61	58	55	74	75	70
9	65	40	15	7	54	58	56	64	67	74

Work with an unstable, dark yellow mutant found in the smooth mucoid stock 500 adds evidence to the increased lability of the dark yellow color gene at high growth temperatures   This unstable variant segregated for both dark and pale yellow colonies   On serial plating of individual colonies it was discovered that all the pale yellow type colonies were stable, whereas the dark yellow type continued to throw a variable percentage of pale yellow colonies on successive platings   Each color type appeared identical, except for color, when grown on nutrient glucose agar at room temperature, but when the plates were incubated at 30 C, all pale yellow colonies were normal in size and appearance, but the dark yellow ones were relatively very small and ridged   A high proportion of the dark yellow colonies contained pale yellow sectors that appeared as typical "bursts "

Dark yellow colonies were suspended in nutrient glucose broth   Aliquots of the same colony were grown in nutrient glucose broth at 3 temperatures   The data's change in proportion of the two types observed is given in table 3   The data show that after 24 hours' growth at high temperature the dark yellow type be

ʼ ᴜ ᴜ ʼ ʼ ɪor one type to replace the other   At 15 C, how-
ever, there is an increase in the proportion of dark yellow types at the 72- and
96-hour sampling, although this trend may not be apparent in the initial stages of
growth   The nature of the instability observed in this stock is not known, but
since it was impossible to obtain a dark yellow stock that remained pure for
color, whereas a pure culture of the pale yellow type could be obtained, it is
believed that this stock contained a mutable gene that became more labile as the
temperature increased   These observations would indicate that the mutable
dark yellow gene became highly mutable at 33 C as compared to temperatures
of 24 C or lower

TABLE 4

*Summary of rate of mutation of stock 400 and of six mutant stocks derived from it when
grown on nutrient broth at 24 C, with calculation of time needed for
mutation to effect a given change in the population*

STOCK	COLONY		TUBES TREATED AS UNITS	COLONIES OBSERVED		MUTATION RATE	GENERATIONS NEEDED FOR MUTATION AT OBSERVED RATE TO EFFECT CHANGE TO MUTANT TYPE	
	Color*	Type†		Total	Mutant		1% change $(q_1 = 0.99)$	50% change $(q_1 = 0.5)$
				$\times 10^3$		$\times 10^{-7}$		
400	Dy	R1	11	224	260	183 0	548	38,200
428	Dy	R4	8	95	32	196 0	505	35,200
441	Dy	S	8	162	96	388 0	257	17,800
491	Wh	S	8	78	5	51 2	1,921	135,000
435	Py	S	8	145	17	2 4	4,100	288,000
446	Py	S	8	212	13	1 2	8,200	576,000
427	Py	R2	8	137	27	133 0	742	52,000

* Dy = dark yellow, Py = pale yellow, Wh = white
† R = rough, S = smooth

IMPORTANCE OF MUTATION AND SELECTION IN EFFECTING CHANGES IN BACTERIAL POPULATIONS

When maintained as stock cultures, most mutants of *P stewartii* were stable,
and variant colony types were seldom found in these stocks even after being
maintained by routine procedures for several months   Occasional mutants were
found, however, that were difficult to maintain as a stock culture because of the
occurrence of a high percentage of undesired colony types, either of the parental
type or of some other variant types   Such instability could be due to a very high
rate of mutation, to mutation with subsequent selection of the mutant, or to
still other factors   To test these possibilities six mutant stocks of strain 400
were selected on which to determine the mutation rate and the mutant's ability
to compete with its parental stock in nutrient glucose broth

Importance of mutation   Mutation rates of the six selected mutants and of
the parental stock (400) are given in table 4   These rates were determined after

24 hours' growth in nutrient broth at 24 C   There is considerable variation among these rates   Such variation is not unexpected if mutation is considered random in nature, particularly since the determination of each rate is based on a relatively small number of colonies

To readily observe the change from a culture of one type of organism to a mutant type necessitates a mass change in that population   For example, in the mutation studies of culture 441 an average of about 1,700 colonies would need to be examined before a mutant colony would be observed, yet after 441 is maintained as a stock culture for 3 months (3 mass transfers each followed by 48 hours' growth at room temperature and storage for 30 days in a refrigerator at 7 to 10 C), it is not unusual to observe more than 50 per cent of the colonies of a mutant type   Can mutation of the order observed for these stocks effect such a mass change in a bacterial population?

The time required for mutation to change one type to some determined proportion of mutant types can be calculated   If

q = proportion of mutant type at time t, and

u = mutation rate,

then the shift in q due to mutation is

$$\frac{dq}{dt} = -uq$$

$$-t = \frac{\ln q}{u} + C$$

$$q = \frac{1}{e^{-u(t+c)}}$$

$$\frac{q_1}{q_0} = \frac{e^{-u(t+c)}}{e^{-u(t+c)}}$$

$$\ln \frac{q_1}{q_0} = u(t_0 - t_1)$$

When $t_0 = 0$

$$t_1 = -\frac{1}{u} \ln \frac{q_1}{q_0}$$

The average rate of all mutation in the parental stock 400 is about $1\ 81 \times 10^{-4}$ (table 4)   The time required for mutation to change 50 per cent of the cells of the culture from parental type to mutant type would be $t = -\frac{1}{0\ 000,018} \ln \frac{0\ 5}{1\ 0}$ or 38,200 cell generations   With this organism about 2,120 days of growth in the logarithmic growth phase would be required for this number of generations to occur   The number of generations required for mutation to effect a change from the parental type to 1 per cent and to 50 per cent of mutant types for each of the stocks studied is included in table 4

If reverse mutation (from mutant back to normal type) were considered

required for mutation to effect an observable change of type in a culture   Some influence, other than mutation, must be acting on a population to account for the change of type observed to occur in cultures such as 441 when carried as stock cultures

Selection within mixed populations   If known proportions of two readily differentiable strains of bacteria were mixed together and the proportions of each type followed by subsequent platings, the interaction of one type with the second could be measured   A change in the relative frequency of one type of individuals

TABLE 5

Changes in bacterial populations when known proportions of the mutant and parental stocks are grown together

MUTANT	PROPORTION OF PARENTAL TYPE COLONIES (STOCK 400) AFTER INDICATED DAYS OF GROWTH										
	0	1	2	3	4	6	8	10	12	14	17
	%	%	%	%	%	%	%	%	%	%	%
427	39	43	54	52	64	73	92	97	99	98	98
428	17	16	23	54	44	87	94	99+	99	100	99+
435	60	51	56	62	48	76	87	87	93	94	99+
441	33	68	87	92	93	97	99	97	100	99	99+
441	29	23	90	99	99+	99+	100	100	100	—	—
446	71	89	99	99	100	100	—	100	—	—	—
491	53	64	73	87	89	97	91	95	97	96	98
491	49	54	44	70	70	86	92	86	64	97	97

in respect to the second type would indicate selective growth, and the intensity of selection could then be measured

The mutants used in the preceding section were initially selected to be readily differentiated from the parent strain (400) by their colony morphology on agar Each mutant was mixed with stock 400 and the proportion of each type followed by plating at desired intervals of time after growth in nutrient broth at 24 C Data for these platings are given in table 5   No mutant tested grew better than did 400, the parent stock   Relative competitive ability of the various mutants is roughly indicated by the rate at which change in the two types occurs   When stock 400 was mixed with any of the variants tested in this experiment, replacement of the variant type by the parental 400 stock was generally rapid and in two cases complete   The average proportion of the parental type present in the initial inoculum was 47 per cent, and after 10 days' growth it had increased to 94 5 per cent   This change occurred in less than 180 cells generation   For

mutation to effect this change at the rate $u = 0\ 0001$ would require 5,560 genera tions, as determined by the formula developed in the preceding section   Obvi ously individuals of strain 400 are reproducing more rapidly than those of the mutant stocks, thereby increasing the comparative frequency of the 400 geno- type   Selection in a bacterial population may be considered equivalent to genic selection, as discussed by Wright (1931), if one assumes that bacteria are asexual organisms dividing by mitosis

As shown in table 5, selection pressure is low until the logarithmic phase of growth is over, then selection pressure increases very markedly   Also it is apparent that selection pressure against the different mutants varies   This is shown in other selection experiments summarized in table 6, in which growth in several environments is considered   Stocks adapted in one environment

TABLE 6

Proportion of four bacterial types present after growth in mixed culture
Plated at 48-hour intervals, data average value of 2 tubes

ADDITIONS TO NUTRIENT BROTH	TEMP	HOURS OF GROWTH	PROPORTION OF RESPECTIVE TYPE				DEATH OF CULTURE OBSERVED
			500*	520†	400‡	427§	
	C		%	%	%	%	hours
—		None	54	25	19	2	
None	24	606	100	0	0	0	>798
1% glucose	12	798	1	0	27	72	>798
1% glucose	24	462	0	0	100	0	500
1% glucose	36	272	0	100	0	0	300
1% glucose + 5% NaCl	24	798	0	0	52	48	>798
1% lactose	24	798	47	0	44	9	>798
10% glucose	24	366	78	4	20	2	375

* Large, mucoid, smooth, yellow colony
† Small, mucoid, smooth, white colony   Mutant of 500
‡ Small, rough, type 1, nonmucoid, dark yellow colony
§ Rough, type 2, nonmucoid, pale yellow colony   Mutant of 400

may be entirely unadapted in a second environment   This phenomenon is shown best by stock 520, which in these mixtures was unable to compete in any environment except 36 C   This temperature is near the maximum at which growth will occur

DISCUSSION

The origin of variation and the interaction of the variant with the parental type are distinct and separate problems of bacterial variation so closely inter related that it is difficult to separate one from the other   It is recognized that in this study the two problems have not been entirely separated, but the methods used have allowed little possibility for one to influence the other   A formula to determine the number of generations for mutation to effect a certain change in a population has been developed   It has been shown that mutation alone is so infrequent as to be ineffective in causing a rapid mass change in a population

tion of the mutation rate by the method used selection may be ignored, since selection has been shown to be relatively ineffective in changing the frequency of a genotype until the logarithmic phase of growth has been exceeded   In determining each of these factors—mutation or selection—small corrections could be made for the factor not studied   However, to do so would necessarily complicate the formula developed and in these cases would not affect the conclusions drawn

Mutation and selection have been shown to be two very important factors in the evolution of bacterial populations   Mutations occur during growth at rates of the order observed in higher organisms   Although the nature of bacterial inheritance is still uncertain, the heritable material or genes must be duplicated and divided before cell division takes place   When the parental genes are not exactly reproduced in two daughter cells, mutation occurs

This study has shown approximately a 10-fold increase in mutation rate when two stocks of *P s'ewai tii* were grown at a temperature of 36 C as compared with 12 C   Intermediate growth temperatures had an intermediate effect on mutation rate   It is interesting to note that the physical forces that influence mutation in high organisms are also very effective in changing mutation rate in bacteria

Once variation is achieved, whether by mutation in a pure culture or by mixture of types, selective forces may act upon the different genotypes   In these experiments the ability of six different mutants to compete with their parent stock was determined   In nutrient broth none of these variants was as adaptive as the parent strain, yet it is conceivable that variation which is nonadaptive in the nutrient broth environment might be adaptive in some different environment   Essentially this possibility was observed in a comparison of four different strains grown together in several environments   One strain completely replaced all others when grown at high temperatures, yet was markedly less adaptive than the other strains in all other environments tested   Had such a mutation occurred in a culture growing in this particular environment, it could be expected that this mutant eventually would have become the predominant type, occurring in any of the other environments this same mutation would have been lost because other types were more adaptive

In higher organisms most mutations are deleterious or nonadaptive   If most bacterial variation is nonadaptive, it is expected that the greatest amount of variation will be found shortly before maximum growth of the population has occurred on a given media and environment   In any environment the least adapted genotypes are lost or occur at a low frequency, whereas those genotypes more adapted increase in frequency   After environmental changes, formerly suppressed types of mutants that arise during growth may replace types adapted in the earlier environment   Stock cultures that have been grown on a certain medium for a long time would not be expected to show sudden changes in their distinguishing characteristics as frequently as freshly isolated cultures or as old

stocks grown under different environments, because selection for a type adapted
to grow on the stock media would already have occurred, whereas selection after
change to a new environment may cause rather wide shifts in characteristics
before stabilization takes place

When a culture is observed at intervals over a period of time, there may be a
gradual transformation of one cultural character into another   The concept of a
gradual change is one that is common in bacteriological literature but one that
often is interpreted as a phenomena in which all or most of the individual cells
making up the culture change together in a definite direction   In the work
discussed above evidence has been presented to show that variation originates as
mutation of normal cells, at a rate probably characteristic for each strain of
bacteria in any specific environment, and selection of types better adapted to
that environment may then take place   The gradual change in the characteris
tics of a culture then becomes one of changing the frequency of occurrence of the
individual cells of each specific genotype, the aggregate of which makes up a
culture

There is much evidence to support the view that evolution in bacteria is
controlled by forces similar to those known to affect evolution of the higher
organisms   In this paper evidence has been given that mutation and selection
are important forces in changing bacterial populations, mutation being the
source of genetic variation upon which selective forces may be effective   After
variation is provided, evolution may proceed subject to selective forces   Under
this view the static nature of a population implied by the term "pure culture" is
misleading and highly problematic a few generations after a single cell is isolated

SUMMARY

The mutation rate ot two stocks of Phytomonas stewartii was determined at
five growth temperatures, 12, 18, 24, 30, and 36 C   Mutations were observed in
colony color, surface appearance, and size   The rate of mutation at 36 C was
approximately 10 times greater than the rate at 12 C, with intermediate mutation
rates being observed at intermediate growth temperatures   The two stocks,
similar in appearance except for colony color, one being dark yellow and the
other pale yellow, differed significantly in their characteristic mutation rates
The kind and pattern of mutation were similar in the two stocks and at the test
temperatures   The temperature coefficient (Q_{10}) of the dark yellow stock was
about 5 0 and of the pale yellow stock approximately 2 5   Certain characters
became very mutable at growth temperatures of 30 to 36 C
The mutation rate of six stable mutants derived from the dark yellow stock

The formula $t = -\dfrac{1}{u}\ln\dfrac{q_1}{q_0}$ was derived to show the generations necessary for a given mutant type to increase to a given proportion if the change in types were due to mutation alone   At the highest mutation rate observed in these stocks 250 generations are needed for mutation alone to effect a 1 per cent increase in a mutant type

Selection as a force in changing frequency of occurrence of a given type in a bacterial population was studied with mixtures of two or more morphologically distinct stocks   By starting with known proportions of each type the change in the proportion of these types could be followed during growth by plating at the desired time intervals   Rapid shifts in the occurrence of types were observed indicating that selection may be a strong force in changing bacterial populations

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Recently, podophyllin, which is a resin of "podophyllum" and similar in many respects to colchicine, has been used in cytological research   Sullivan and Wechsler (1947) report the similarity of the action of podophyllin to that of colchicine on young, growing root tips of *Allium cepa*   The spindle mechanism was evidently impaired, and pronounced cytological effects were noted in the late prophase   The present report is concerned with the effect of podophyllin on two strains of *Eberthella typhosa*

It was of interest to note whether there would be any changes in the colonial character of two strains of *Eberthella typhosa* when exposed to a saturated solution of podophyllin in nutrient broth

The one strain of *Eberthella typhosa* used in this study produced typical S type colonies on nutrient agar   A single cell of this culture was isolated and put into nutrient broth for a previous study concerning the effects of X-rays on this strain (Grainger, 1947), as well as for this study   The strain was characteristic of the species in respect to all the biochemical and physiological characteristics as described in *Bergey's Manual* (1939)

The other strain of *Eberthella typhosa* used produced typical R type colonies on nutrient agar   It had been isolated recently in another study and was characteristic of the species in respect to all the biochemical and physiological characteristics as described in *Bergey's Manual* (1939), except for one difference—this strain would not ferment the sugar galactose

The resin of podophyllum (Merck) used in this study was found to be only slightly soluble in water and gave a light brown color to the solution   A small amount (1 gram) was added to each of two flasks that contained 100 ml of nutrient broth   This amount allowed for a well-saturated solution of podophyllin in the broth   The reaction was adjusted to pH 7 0, and the material was then sterilized

One loopful of a 24-hour nutrient broth culture of the S strain of *Eberthella typhosa* was placed in the flask of nutrient broth containing the podophyllin   One loopful of the same S strain was also added to a flask containing 100 ml of nutrient broth   This served as a control   The same procedure was followed with the R strain of *Eberthella typhosa*   The flasks were then placed in the incubator at 37 C

Subcultures were made daily on nutrient agar plates by the streak method from the flasks containing the S and R cultures with the podophyllin in the nutrient broth, as well as from the flasks of nutrient broth which served as the controls   The colonies were studied by means of a colony microscope lens (3×) to note any changes in morphology   At least 100 well-isolated colonies were

studied daily on the nutrient agar obtained from the subcultures from each flask for a period of 30 days   There was no difference noted in the colonies from either the S or the R cultures of *Eberthella typhosa* in the flasks containing the podophyllin in nutrient broth, as compared with the S and the R colonies from the control flasks of nutrient broth   Occasionally, however, an inter mediate form was observed from the S culture from the flask containing the podophyllin, but this was also observed from the control broth   There was no difference in the colonial character noted from the R culture in either the test flask or the control

SUMMARY

The resin of "podophyllum" (podophyllin) saturated in nutrient broth did not have any effect on the colonial character of a S or R strain of *Eberthella typhosa*

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AGAINST NINE SPECIES OF BACTERIA

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Each addition to the ever-lengthening list of antibiotic substances increases considerably the difficulty of identifying them short of isolation in pure form Methods of identification applicable to extracts, culture filtrates, and concentrates are needed because the substances are first obtained in these forms, isolation of the pure substance may require long and tedious work with relatively large amounts of material

During the work in this laboratory, which led to the isolation and characterization of about ten new antibacterial substances, it was necessary to devise a procedure which would enable us to decide whether or not we were working with new substances

The data in the literature appeared ample to enable us to identify an unknown substance by measuring its activity against several bacteria and comparing the activities with those reported for the previously described antibiotic substances Frequently, however, several strikingly different activities against the same species of bacteria were reported for the same substance   When one value was ten times another, it was impossible to decide which was the correct one   Since the details of the test method, composition of the test media, concentration of the bacteria, strain of bacteria, temperature, and duration of incubation all influence, to varying degrees, the activities found for a substance, these inconsistencies could be explained   Some authors, however, gave either no information or incomplete information about their test methods   To attempt to identify an unknown with one of the known antibacterial substances, one might try to use the procedures of each author—an impossible task—or to obtain as many as possible of the known substances and to use them to compile a consistent body of data using one technique with a few selected strains of bacteria, as has been done here   No attempt has been made to compare the results obtained here with those in the literature because of the differences in technique, strains of bacteria used, composition of test media, and temperatures of incubation The activities of many of the antibacterial substances that have been isolated from green plants and fungi are collected in a recent review (Kavanagh, 1947*b*)

This paper reports the activity of 17 naturally occurring and 5 synthetic antibacterial substances against 9 kinds of bacteria, as determined by serial dilution methods   Details of the antibacterial methods are given elsewhere (Kavanagh, 1947)   Frequently more than one active substance occurs in the culture filtrate or concentrate, usually such a mixture must be resolved into

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its components before they can be identified   The active material in a solution
suspected of containing a new antibacterial substance was fractionated into
acidic, neutral, and basic groups before an attempt was made to identify them
Such simple chemical tests as color reaction with ferric chloride, thermal and
pH stability, and susceptibility to inactivation by clarase or penicillinase were
made   The antibacterial activities of the fractions were then measured against
the 9 species of bacteria   The activities against the other bacteria relative to
that against *Staphylococcus aureus* were computed and compared with the rela
tive activities obtained for the 17 naturally occurring antibiotic substances
The antiluminescent activities (Kavanagh 1947a) were also determined

The values in table 1 are those obtained in the majority of tests, although a
single measurement is unlikely to be less than one-half as great or more than
twice as great   The reproducibility of the values is a property not only of the
antibacterial substance but also of the species of bacteria   The measured activi
ties are sufficiently consistent for purposes of identification, since no single
measurement determines the identity of a substance

Ba teria   The bacteria used were *Bacillus mycoides* ATCC 9634, *Bacillus
subtilis* ATCC 9633, *Escherichia coli* ATCC 9637, *Klebsiella pneumoniae* 9997,
Mycobacterium phlei ATCC 10142, *Mycobacterium smegmatis* (smegma) ATCC
10143, *Pseudomonas aeruginosa* ATCC 10145, the Heatley strain of *Staphylococc
cus aureus* ATCC 9144, and *Photobacterium fischeri*, the Doudoroff strain, ob
tained from G Rake

The *B mycoides*, *B subtilis*, and *E coli* were "standard tester strains" of S
A Waksman

The antibacterial substances were obtained from the following   aspergillic
acid from G Rake of The Squibb Institute for Medical Research, citrinin from
J H Bailey of the Winthrop Chemical Company, 4,6-dimethoxy-toluquinone
from Harold Raistrick, dihydrostreptomycin trihydrochloride (M 2216 H2,
about 740 μg/mg) from O Wintersteiner of the Squibb Institute, gliotoxin
from J D Dutcher of the Squibb Institute, helvolic acid (fumigacin) from E
A Doisy of St Louis University, "Hogeboom and Craig No 1" from L C
Craig of the Rockefeller Institute for Medical Research, kojic acid from the
Commercial Solvents Corporation, mycophenolic acid from Harry Sobotka,
patulin, penicillic acid, and spinulosin from Harold Raistrick, the crystalline
salts of the penicillins from the Commercial Solvents Corporation, streptomycin
trihydrochloride (M 2213, 840 μg/mg) from O Wintersteiner, streptomycin
trihydrochloride-calcium chloride double salt (109X28C, 715 μg/mg) and hydro
genated streptomycin trihydrochloride (144X390I, 800 μg/mg) from the Re
search Laboratories of Parke, Davis and Company, and streptothricin (425
units/mg) from R T Major of Merck and Company   The "Hogeboom and
Craig No 1" (Hogeboom and Craig, 1946) was thought by Doering, Dubos,
Noyce, and Dreyfus (1946) to be identical with their "ustin "   The activities
of the dihydrostreptomycin and of the hydrogenated streptomycin were identical

,-nap oquinone was a commercial product and was used as received   The tolu-*p*-quinone was an Eastman product that had been recrystallized   Biformin (Robbins, Kavanagh, and Hervey, 1947*b*), pleurotin (Robbins, Kavanagh, and Hervey, 1947*a*), and cassic acid (Robbins, Kavanagh, and Thayer, 1947) were isolated in this laboratory   The biformin was the purest obtained, the other two substances were crystalline

All the antibacterial substances were assumed to be pure unless there was a statement to the contrary   Except for the streptothricin the amount of impurities in the compounds was too small to affect the activities as measured by the serial-dilution method used here

TABLE 1

Minimum inhibitory concentration of antibacterial substances in micrograms per milliliter

ANTIBACTERIAL SUBSTANCE	B MY COIDES	B SUB TILIS	S AUREUS	E COLI	K PNEU MONIAE	P FIS CHERI	P AERU GINOSA	M PHLEI	M SMEGMA
Aspergillic acid	2	4	4	62	13	1	1 000	125	16
Biformin	13	0 04	0 3	1 7	1 7	0 0?	53	0 6	3 3
Cassic acid	4	8	8	1 000	500	0 25	>250	8	30
Citrinin	32	16	16	>1 000	—	16	—	125	250
Dihydrostreptomycin	0 25	0 5	0 0	0 25	0 13	200	4	0 25	1
4 6-Dimethoxy toluquinone	32	4	1	250	125	2	1 000	32	16
Gliotoxin	0 25	0 25	0 15	25	6	0 25	500	4	4
Helvolic acid	4	16	1	>1 000	4		—	>32	>32
Hogeboom and Craig No 1	1 6	0 8	6	>50	>50	1 6	—	6	13
Hydrogen peroxide	31	4	8	10	5	5	8	31	4
Kojic acid	2 500	620	1 250	2 500	620	2 500	5 000	2 500	310
2 Methyl 1 4 naphthoquinone	12	3	1 7	220	28	3	>400	14	36
Mycophenolic acid	500	250	250	500	>1 000	125	>1 000	500	250
Patulin	16	4	8	8	8	0 25	125	16	1
Penicillic acid	32	8	16	64	64	1	1 000	64	32
Penicillin G	30	0 03	0 016	14	110	16	500	14	450
Penicillin X	30	0 06	0 03	14	240	8	500	29	470
Pleurotin	3	0 2	0 8	>500	>500	6	—	>32	32
Spinulosin	125	125	63	250	250	>16	500	250	500
Streptomycin	0 13	0 25	0 03	0 25	0 13	200	4	0 25	1
Streptothricin	100	0 8	0 1	0 3	0 1	20	2	7	14
Tolu *p*-quinone	4	1	1	25	13	0 06	125	16	4

RESULTS

The values given in table 1 are the minimum concentrations of the substances in micrograms per milliliter that prevented evident growth of the bacteria for 24 hours at a temperature appropriate for each species of bacterium   The values for streptothricin are for the compound as received, correction for impurities was not made   The values for streptomycin and dihydrostreptomycin are computed for the free base   The two samples of streptomycin were equally active, as were the two samples of dihydrostreptomycin

The three species of bacteria generally most sensitive were *B subtilis, S aureus*, and *P fischeri*, a gram-negative bacterium   The absolute activities against *S aureus* ranged from 0 016 for penicillin G to 1,250 for kojic acid, with most of the values less than 16 micrograms per milliliter   *B subtilis* and *S*

aureus were about equally sensitive   *P fischeri* was the most sensitive organism
for six of the antibacterial substances but was the least sensitive to streptomycin
and dihydrostreptomycin   The least sensitive of the bacteria was *P aeruginosa*,
which was inhibited appreciably only by hydrogen peroxide, streptomycin,
dihydrostreptomycin, and streptothricin   The data presented here, as well
as those in the antibiotics literature, indicate that the activity of a substance
against one species cannot be predicted from the knowledge of the activity against
another species of bacteria

The antibacterial substances can be put into two groups those active against
the gram-negative bacteria and those much more active against the gram posi
tive than against the gram-negative bacteria   The EC/SA ratio computed
by dividing the minimum concentration that inhibits *E coli* by that needed
to inhibit *S aureus* provides an index for placing the substance in one of two
quite distinct groups   The naturally occurring antibacterial substances for
which EC/SA $\leq$ 16 seems to include those generally recognized to be active
against the gram-negative bacteria   The naturally occurring substances, for
which data are reported here, that are not in the group active against *E coli*
have EC/SA > 100   The members of a group can be separated from each other
by utilizing the activities against some of the other test bacteria

The activities of the antibacterial substances have been reported as concen
trations measured in micrograms per milliliter   In comparing compounds with
the greatly different molecular weights found among these substances (from 34
for hydrogen peroxide to 581 for streptomycin base), molar concentrations and
not weight concentrations should be compared   Compounds with equally
active molecules but different molecular weights will then have the same activi
ties, whereas a comparison on a weight concentration basis would indicate that
the substance with the lower molecular weight is the more active   For example,
penicillin G and streptomycin are equally active against *S aureus* when molecular
concentrations are compared   Many other such comparisons are possible using
the data in table 1

APPLICATION OF THE METHOD TO IDENTIFICATION OF AN UNKNOWN SUBSTANCE

As an example of the usefulness of the data of table 1 in identifying an anti
bacterial substance, results obtained with the crude culture filtrate from *Penicil-
lium claviforme* may be cited   This filtrate was presumed to contain patulin
(Chain, Florey, and Jennings, 1942, 1944)   Since the concentration of the active
substance[1] was unknown, the antibacterial activities against four bacteria rela
tive to its activity against *S aureus* were computed and are given in table 2

The EC/SA ratio of two, being less than 16, put the unknown substance in
the group active against *E coli*   The naturally occurring members of this
group include aspergillic acid, biformin, patulin, penicillic acid, spinulosin,
streptomycin, and streptothricin   The high relative activity against *B mycoides*
or *B subtilis* eliminated biformin, streptomycin, and streptothricin from con
sideration   The relative activities against *E coli* and *K pneumoniae* indicate

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[1] Only one substance with measurable activity is assumed to be present

The great similarity between the relative activities of patulin and penicillic acid (identical for two bacteria) emphasizes the necessity for considering the activity against all nine bacteria when attempting to identify an unknown substance Antibacterial methods can make identification highly probable, but only chemical methods can make it certain

TABLE 2

Comparison of a filtrate containing an unknown suspected of being patulin with seven known substances

SUBSTANCE	ACTIVITY RELATIVE TO *S aureus* = 1			
	B mycoides	*B subtilis*	*E coli*	*K pneumoniae*
Filtrate	1	0 5	2	1
Aspergillic acid	0 5	1	16	3
Biformin	40	0 1	5	5
Patulin	2	0 5	1	1
Penicillic acid	2	0 5	4	4
Spinulosin	2	2	4	4
Streptomycin	4	8	8	4
Streptothricin	500	1	1	1

DISCUSSION

Since specific chemical tests suitable for application to very dilute solutions or very impure preparations of antibacterial substances are unknown, use must be made of biological methods   The most useful biological property, the one that by definition is common to this chemically heterogeneous group of organic compounds, is the ability to prevent the growth of microorganisms   Detection of the presence of an antibiotic substance by means of its inhibition of a micro-organism is relatively simple because only one organism is required   The use of antibiotic action to identify a substance, however, necessitates the use of several microorganisms and requires a knowledge of the action of all of the known substances against this same set of microorganisms   Furthermore, a stand-ardized technique must be used with the unknown and with all of the identified substances

Too much of the published data on the antibacterial activities are good only for putting a substance into one of two groups, those active against gram-positive bacteria or those active against both gram-negative and gram-positive bacteria (the substances with activity only against fungi are not considered here)   Most of the "bacterial spectra" were made with medically important bacteria in an effort to ascertain possible therapeutic applications of the substances   Fre-quently such lists of bacteria contain few that are available in many laboratory collections, few that are relatively nonpathogenic, and few that might be valuable in identifying antibiotic substances   All of the antibacterial substances of

natural origin have been tested for activity against only one species of bacteria, *S aureus*

The nine species of bacteria used in this work were selected because they grew rapidly in simple media, were susceptible to some of the substances, and differed greatly in their susceptibility to different substances   This is the first use of a species of *Photobacterium* in an antibacterial test, the usual test with it is an antiluminescent one (Rake, McKee, and Jones, 1942, Kavanagh, 1947a)   It proved to be one of the more valuable test bacteria   If these strains of bacteria are employed in the test procedures devised for them (Kavanagh, 1947), other workers should be able to obtain the activities given in table 1, thus eliminating the necessity of actually determining the activities of all of the known substances each time an unknown is identified

If only one substance is to be identified, chemical purification and identification possibly would be less time-consuming than the antibacterial method

SUMMARY

The activities of 17 antibacterial substances of natural origin and 5 synthetic ones were measured against 5 gram-positive (2 acid-fast) and 4 gram negative species of bacteria

Bacillus subtilis, *Staphylococcus aureus*, and *Photobacterium fischeri* were the most sensitive, and *Pseudomonas aeruginosa* was the least sensitive, of the nine bacteria

The tentative identification of patulin in a culture filtrate is given as an illustration of the application of the antibacterial method

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~ ~~ ` ' ..TIONS IN STAPHYLOCOCCUS AUREUS BY CHEMICAL TREATMENT OF THE SUBSTRATE

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The literature on the induction of mutations in microorganisms by irradiation might well begin with the report by Henri (1914) on the appearance of two new forms of the anthrax bacillus subsequent to exposing these organisms to ultraviolet light   That Henri appreciated the significance of his finding is evident from his conclusion, "La lumière apparaît donc ainsi comme un agent fondamental de l'évolution intervenant par l'attaque plus ou moins profonde des fonctions nutritives intimes de la cellule "   However, since many of the implications of studies on bacterial mutations are apparent only by liberal use of analogy to the genetics of higher forms, similar studies were necessarily retarded awaiting the development of the modern concepts of gene mutations   The recent interest in bacterial genetics has resulted in excellent reviews on this subject (Luria, 1947, Braun, 1947)   One observes that in addition to radiations a variety of other methods are available for the production of mutations   Auerbach (1945) demonstrated that mustard gas would produce mutations in *Drosophila melanogaster*, and a similar treatment of microorganisms with the nitrogen or sulfur mustards, acenaphthene, and other chemical agents has resulted in the enhancement of the mutation rate

Many of the reports on mutations in microorganisms are concerned with selection of naturally occurring mutants from the population   For example, Pinner and Voldrich (1932) observed the production of occasional nonpigmented colonies of *Staphylococcus aureus*, when a strain of that culture developed from a single cell and grown in nutrient broth was streaked on nutrient agar   If the organisms were transferred routinely in nutrient broth containing 5 per cent pleural fluid with a high agglutinin titer for the *S aureus*, the culture would finally appear to be almost a pure *Staphylococcus albus*   In some cases it is difficult to decide to what extent the factors of selection are operative   The mutations in aspergilli reported by Thom and Steinberg (1939) and Steinberg and Thom (1940) may involve selections, or they may be due entirely to induced mutation   By the addition of a wide variety of agents to the medium these authors consistently found mutations with aspergilli   Nitrite, in the acid medium used for molds, was particularly active in producing large numbers of

mutant forms　These mutants were stable over a long series of transfers on normal media　The types of mutations observed were similar to those occurring spontaneously, the number of mutants, however, was increased tremendously by the addition of various substances to the medium　Most of the substance employed were selected because they reacted with the amino groups on proteins The authors suggested that the chemicals reacted with the protein components of the genetic mechanism and showed that some reversion occurred when the mutants were grown in a medium containing excess d-lysine

Using another approach, Stone, Wyss, and Haas (1947) increased the resistance of S aureus to penicillin and streptomycin by irradiation of the substrate prior to inoculation　Several lines of evidence were presented in an attempt to rule out selection as the determining factor in these experiments　They suggest that modified substrate molecules may be assimilated by the organism and built into inexact replications of the genetic mechanism　To test this theory we have treated the substrate with a number of chemical agents and have measured the effect of such treatment on the mutation rate of S aureus　In most

TABLE 1

Increase in mutation to penicillin and streptomycin resistance by treatment of broth with hydrogen peroxide one hour prior to inoculation

TREATMENT OF BROTH	TOTAL COUNT	PENICILLIN 0 05 UNITS/ML		STREPTOMYCIN 3 UNITS/ML	
		Colonies	Mutants/ million	Colonies	Mutants/ million
Control	470,000,000	2,300	4 9	8,800	18 7
6 ppm H₂O₂	330,000,000	50,000	151	230,000	696

cases unstable chemicals were used to treat the substrate so that at the time of inoculation no residuum remained to obscure the result by possible direct action upon the organism

EXPERIMENTAL

The methods employed involved the detection of penicillin- and streptomycin resistant mutants in S aureus　They are essentially identical with those reported by Stone et al (1947)　The substrate, usually nutrient broth, was treated with the chemical agent and permitted to stand at room temperature for 1 hour If tests indicated the disappearance of the agent, the medium was inoculated with about a million cells per ml from a young broth culture　Residual hydrogen peroxide was checked qualitatively by starch-iodine, by catalase, and by the titanium sulfate method of Bonet-Maury (1944)　The latter method was employed with the Coleman spectrophotometer, it permitted quantitative measurements to 0 1 ppm, but in the presence of broth it was somewhat less sensitive After incubation the assay for resistant mutants was made　The experiment reported in table 1 shows the plate counts when organisms are grown in untreated broth and in broth treated with 6 ppm hydrogen peroxide 1 hour before inoculation

or untreated broth were able to form colonies, of the organisms grown on the broth treated with hydrogen peroxide 50,000 out of a total population of 330 million per ml were able to grow   Thus the number of mutants per million was increased over 30 times   A similar situation exists with the streptomycin mutants   A number of colonies were picked from the plates made from the treated broth and the drug resistance was shown to persist even after serial transfer on plain nutrient agar   These organisms were no more resistant to peroxide, nor did they grow faster in the peroxide-treated broth, than the control organisms

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TABLE 2

Effect of concentration of hydrogen peroxide and other oxidizing agents on the mutation rate

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TREATMENT OF BROTH		MUTANTS PER MILLION	
		Penicillin	Streptomycin
		0 05 units/ml	*3 units/ml*
Control		8 4	26 7
H₂O₂	9 ppm	no growth	no growth
H₂O₂	3	400 0	1334 0
H₂O₂	1	26 7	84 0
H₂O₂	3	10 0	23 4
Cl₂	125	no growth	no growth
Cl₂	75	10 0	30 0
Cl₂	6	12 0	23 0
I₂	6	2 5	10 5
NaNO₂ 500		—	25 0
KMnO₄ 10		20 0	36 0
Control		8 0	33 4
Aerated with oxygen		7 0	30 0
Irradiated 15 minutes (ultraviolet light)		180 0	700 0

Although at the time of inoculation no peroxide could be detected in the broth treated with 6 ppm H_2O_2, experiments on concentration were instituted to observe whether or not mutations would be induced by treatment of the broth with concentrations well below the amount inhibiting growth   As shown in table 2 the bacteria failed to make visible turbidity in 18 hours in broth treated with 9 ppm H_2O_2 However, even the treatment with 3 and 1 ppm peroxide resulted in a definite increase in the mutation rate

The addition of 6 ppm Cl_2 or I_2 failed to produce any increase in the mutation rate   In fact, when the concentration of Cl_2 added to the broth was increased to a value just short of that which gave a free chlorine residual and thus prevented growth, the mutation rate to streptomycin and penicillin resistance still remained essentially that of the control   High concentrations of $NaNO_2$ failed to increase the mutation rate to streptomycin resistance (penicillin not tested), although in this case much of the nitrite remained in the broth at the time of inoculation   The nutrient broth used was of a neutral pH, so the reaction with

amino acids suggested by Steinberg and Thom could not be expected to occur in this experiment   Potassium permanganate added at a level which reacted completely with the broth failed to affect the mutation rate   Bubbling pure oxygen gas through the medium for 1 hour prior to inoculation did not have any effect on the mutation rate of organisms subsequently inoculated therein   These experiments suggest that the effect of hydrogen peroxide on the mutation rate is fairly specific and not merely the result of growing organisms in a medium with a high oxidation-reduction potential

The time elapsing between the addition of 6 ppm of peroxide to the broth and inoculation was varied from 15 minutes to 22 hours without markedly affecting the result (table 3)   Within experimental error the mutation rate was increased about 5- to 10-fold in the case of penicillin and 10- to 20-fold in the case of streptomycin   From these data it appears reasonable that the effect of the hydrogen peroxide is due to its reaction with some component in the medium

TABLE 3

The effect of time elapsed between treatment of the broth with 6 ppm hydrogen peroxide and inoculation

TIME BEFORE INOCULATION	MUTANTS PER MILLION	
	Penicillin	Streptomycin
	0 05 units/ml	*3 units/ml*
15 min	73 5	541 0
40 min	97 5	795 0
2 hours	74 8	621 0
3 hours	64 0	848 0
5 hours	60 3	578 0
22 hours	42 7	331 0
Control (no H_2O_2)	9 4	46 4

In order to determine whether or not a selective action of the treated broth was responsible for the result the rate of appearance of the mutants in the young culture was studied   Platings made at 0, 3, 6, and 24 hours after inoculation indicated that in the peroxide-treated broth the mutants appeared at a rate that could best be explained by assuming that the mutations were induced by treated substrate   Very careful measurements on growth rates of mutant subcultures, of the parent strain, and of mixtures of the latter with mutant cultures indicate that in neither normal broth nor peroxide-treated broth did population change occur which would permit attributing the results to a selective action

Several chemical substances have been treated with hydrogen peroxide and then added to normal broth   For example, 100 mg of phenyl alanine were dissolved in 100 ml of water to which 100 ppm hydrogen peroxide were added   After an hour one ml of this mixture was added to 50 ml of broth and inoculated with *S aureus*   After a suitable growth period the resistant mutants were determined and compared with results obtained with control cultures   The results show a considerable enhancement of the mutation rate   Much of the

residual peroxide acting on the broth components   ....y ' '' effect was due to the

Other substances giving the increased mutation rate when treated with peroxide are tryptophane, tyrosine, adenine, uracil, and guanine   Tryptophane is reported to be converted to indole acetic acid by the action of hydrogen peroxide or ultraviolet light, but the addition of indole acetic acid to the medium had no effect on the mutation rate   A number of reducing agents such as thioglycolic acid, sodium sulfite, and sodium sulfide had no effect on the rate of mutation

Stahmann and Stauffer (1947) treated fungous spores with methyl-*bis*-(*β*-chloroethyl)-amine and obtained a high mutation rate measured by colonial variation   The concentrations used (0 01 M) killed a large percentage of the mold spores in 30 minutes and showed a pronounced increase, not only in the fraction of the survivors which were mutants, but in the total number of mutants in the smaller surviving population   We employed *tris*-(*β*-chloroethyl)-amine, which at equivalent molar concentrations showed about the same killing rate with *S aureus* cells as the methyl derivative used by Stahmann and Stauffer exhibited with the mold spores   When 90 per cent of the *S aureus* cells were killed, the rate of occurrence of penicillin-resistant cells was found to have increased 20-fold   When this substance was added to the broth at several concentration levels and permitted to react for 4 hours before inoculation, it resulted in a pronounced increase in the number of penicillin-resistant and streptomycin-resistant cells in the resulting population   It is believed from lack of odor and inhibitory action of the broth at the time of inoculation that the mustard had completely hydrolyzed before the cells were added   The action here, also, appears to be one of mutation induced by action upon the substrate

The correlation between the action of hydrogen peroxide and ultraviolet light is difficult to determine   Irradiation of water by ultraviolet under the conditions of our experiments produces a considerable amount of peroxide   Similar irradiation of the broth produces no detectable residual peroxide since it appears to react quickly with broth constituents   Experiments in which catalase was added to the broth during irradiation and after treatment with hydrogen peroxide gave conflicting results

SUMMARY AND CONCLUSIONS

These data indicate that treatment of broth with ultraviolet light is not a unique indirect method of inducing mutations   Hydrogen peroxide reacts with some broth components, and an increase in mutant forms appears when organisms are grown in their treated medium, although no peroxide remains at the time of inoculation   A similar action occurs with a nitrogen mustard

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TO THEIR AGE

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The relationship between age and immunity has long been recognized The "diseases of childhood" and the Shick and Dick susceptibility curves in the young furnish illustrations of this relationship   Hirszfeld, (Halber *et al*, 1927) proposed the theory that certain so-called "normal" antisubstances (e g , isohemagglutinins) are inherited, and these become functionally mature at some specific time in the individual's development—a characteristic referred to as "serological maturity "   Baumgartner (1934) has reviewed the literature on the relationship of age to immunological reactions through 1933 and has given evidence to substantiate Hirszfeld's hypothesis   Kahn (1936) demonstrated that the localizing power of the cutaneous tissues for protein antigens possessed by immune adult rabbits is much more marked than the similar capacity of young rabbits

Wendt (1925) claimed that, though the majority of sera from cattle were Wassermann-positive (icebox fixation), calf sera were usually negative   Mackie and Watson (1926) observed that the sera of adult cattle, sheep, and rabbits, with few exceptions, gave positive reactions with the Wassermann (37 C fixation) or Sachs-Georgi tests, but the sera of some calves, lambs, and pooled young rabbits were usually negative   Sherwood, Bond, and Clark (1941) and Kemp, Fitzgerald, and Shepherd (1940) have reaffirmed these observations and reported similar findings with beef, dog, and sheep sera

In the experiments to be described serologic studies with sera from rabbits were made in relation to the age of the animals   Newborn rabbits had their birthdays tattooed on their ears and were first tested serologically when they became about 45 to 55 days old   They were then retested at intervals of 14 to 20 days until five or more examinations had been made   The weight of the animals when first tested was about one pound, and the blood was obtained by cardiac puncture   The Kahn standard and differential temperature tests (Kahn, 1946) were employed, and they were performed on unheated portions of serum as well as on portions heated at 56 C for 30 minutes

Thirty-one animals, including 10 females and 21 males, were used in the study The results in nine representative cases are included in table 1   The tabulated results of the differential temperature tests include only the reactions obtained at 1 C   The reactions obtained at 37 C were found to be essentially negative and were not included in the table

RESULTS

It will be noted from the representative cases listed in table 1 that all animals gave negative flocculation reactions with the standard Kahn test when first

TABLE 1

Increase in potency of serologic reactions in rabbits with age

AGE	KAHN REACTION		DIFFERENTIAL TEMPERATURE REACTION (AT 1 C)	
	Heated serum	Unheated serum	Heated serum	Unheated serum

Rabbit 497 (female)

Days
52
61
82
101
131

Rabbit 481 (female)

Days
51
63
81
103
130
158
170

Rabbit 148 (female)

Days
56
68
86
108
135

Rabbit 182 (female)

Days
51
63
77
100
...

Rabbit 491 (male)

52
64
82
104
131
145
157
160

Rabbit 498 (male)

50
62
80
102
120

Rabbit 487 (male)

43
55
63
85
111
128

Rabbit U493 (male)

52
61
82
101
131

Rabbit 478 (male)

50
68
80
108
135

The symbols ++++, +++, ++, +, and ± represent the usual degrees of flocculation
• Quantitative titers are given in parenthesis

examined at ages of approximately 45 to 55 days When unheated serum, instead of serum heated at 56 C, was employed, the same test gave negative results in all instances except one in which a very weak reaction was noted When the tests were conducted at 1 C with heated serum, again only one rabbit gave a weak flocculation reaction, with unheated serum, however, two definite flocculation reactions were obtained It would appear that the sera of rabbits under 2 months of age show little or no serologic reactivity with lipid antigen

On successive examinations the Kahn standard test did not begin to give positive reactions until the rabbits were approximately 3 months of age But Kahn tests with unheated sera began to give positive reactions at about 2 months of age Reactions appeared earliest, remained most consistent, and reached their highest quantitative levels in the case of the unheated sera tested at 1 C

Seven of the 31 rabbits tested showed reactions typified by rabbit 491, i e, a persistently negative Kahn reaction in the standard test, weak fluctuating reactions with heated sera at 1 C, and fairly consistent flocculation with un heated sera both at room temperature and in the cold The majority of animals showed the patterns illustrated by rabbits 148, 498, 487, and U493 in the table, beginning with negative reactions when under 2 months of age, approaching positivity between 2 and 3 months, and remaining positive after that time A few of the animals showed patterns which varied somewhat from the above, illustrated by rabbits 497, 478, 481, and 482

SUMMARY

Data are presented showing that Kahn reactions given by rabbits tend within limits to become stronger with the increase in age of the animals Rabbits under 2 months of age are generally sero-negative The appearance of positive reactions after 2 months is relatively common Unheated sera tend to give stronger Kahn reactions than sera that have been previously heated at 56 C for 30 minutes Highest quantitative titers are obtained when the tests are con ducted at a low temperature (1 C) with unheated sera

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Many theories have been proposed to explain the acid-fast property of the tubercle bacillus, but no satisfactory explanation has yet been given   It is suggested that the property is dependent upon the permeability of the cytoplasmic membrane   Evidence will be presented in support of this concept It will be shown that when the Ziehl-Neelsen technique is employed the dye exists within the cell in two distinct portions   a small portion is bound to the cytoplasm and the remainder is free   The characteristic color of the stained bacillus is due to the free dye which can be removed without altering the acid-fast property   In the past, investigators (Koch, 1897, Aronson, 1898, 1910, Tamura, 1913, Anderson, 1932) have believed that this phenomenon of acid-fastness might be based upon the properties of chemicals isolated from the tubercle bacillus or of complexes of these chemicals   At one time there was a general belief that the presence of a wax sheath around the cell was responsible for its peculiar staining characteristics, but this is no longer tenable after the study made by Knaysi (1929)   Some authors including Ehrlich thought that acid-fastness was related to the permeability of the cell membrane, but this structure was not clearly defined and experimental evidence was not given The one point on which there is complete agreement is that the integrity of the cellular structure must be maintained to preserve the acid-fast property

Since our proposed explanation of the mechanism of acid-fastness is based on the function of a cellular structure, a brief review of pertinent information on the cytology of the tubercle bacillus is indicated

The cell wall of the tubercle bacillus has been observed directly with the electron microscope by Mudd and Anderson (1944)   Mudd and Mudd (1927) demonstrated the hydrophobic property of the surface of the bacillus   The apparent functions of the cell wall are to protect the cell from mechanical injury and to impart to the cell its characteristic shape

The electron microscopic studies of Mudd, Polevitsky, and Anderson (1942) and Mudd and Anderson (1944) give direct evidence of the cytoplasmic membrane in bacteria   According to Knaysi[1] (1929, 1938, 1944, 1946), who refers frequently to this structure, the membrane of the tubercle bacillus probably consists of lipids and protein   With ordinary technique it appears to be the external boundary of the cytoplasm rather than a separate structure

EXPERIMENTAL RESULTS

Although there is some evidence that the cell wall of the tubercle bacillus retains small amounts of certain other dyes, our observations suggest that this

[1] A recent personal communication from Knaysi states that what he referred to as the cell "membrane" in his paper (1929) is now usually known as the cytoplasmic membrane

structure does not stain acid-fast to a degree that would add to the color of the bacillus as seen by the ordinary light microscope   The following observation indicates that the portion of the cell which is acid-fast-staining is within the cell wall

Cells from a young,   tively growing culture of tubercle bacilli are stained solidly by the Ziehl-Neelsen technique   Later, because of prolonged unfavorable environmental conditions, granular forms appear   In these older bacilli the cytoplasm separates into granules that vary in size and number and stain strongly acid-fast   Ordinarily no stained material is seen between the granules, but occasionally, when separation of the cytoplasm is incomplete, a faintly stained line may be seen connecting two granules   We believe that when the separation of the cytoplasm is complete the cytoplasmic membrane surrounds the individual granules and is no longer continuous over all the granules in a single bacillu When a granular, cell is stained first by the Ziehl-Neelsen method and then outlined by nigrosine, a white continuous border is seen surrounding the acid fast cell   This represents the unstained cell wall which retains the granules and prevents the nigrosine from outlining the individual granules (figure 1)

It is our belief that when the Ziehl-Neelsen technique is employed the dye gains entrance into the interior of the cell through the cytoplasmic membrane, where a small portion of the dye is firmly absorbed by the cytoplasm and can be removed from the cell only with difficulty   The remaining free dye is held in the cell because it is unable to diffuse through the cytoplasmic membrane, and it is to this free dye that the characteristic red color of the stained bacillus is due

If the greater portion of the dye in the cell can be shown to act as though it can be precipitated and then redissolved, it is evidence that the dye behaves as free dye   When a film of tubercle bacilli, stained with either the acetate or hydrochloride of rosanilin to which 0 5 per cent of sodium chloride is added, is examined microscopically, the dye appears to be evenly distributed throughout the cells and of uniform density (figure 2)   The free dye in these stained bacilli can be precipitated and redissolved by the following process, which may be observed microscopically

The application of acid alcohol to the film causes the color of the bacilli to change to a bluish red   If the film is now washed with water, there will be noted a sudden shift of the dye to certain points in the cell, resulting in the formation of one or more red-black bodies (figure 3)   These bodies, which are referred to as beads, are often considerably greater in diameter than the width of the cell The areas between the beads stain faintly, and the width of the cell in these areas is diminished   These faintly stained portions contain the dye that is absorbed by the cytoplasm

The accumulated or precipitated dye which forms the beads can be dissolved almost instantaneously by the application of either 5 per cent phenol or 95 per cent ethyl alcohol, following which the dye spreads evenly throughout the cell and gives the cell a uniformly bright red appearance (figure 4)   The process of accumulation and redistribution of the dye may be repeated in the same cell without restaining, but eventually beads will fail to form   Apparently

en the concentiation of the dye is reduced the beads decrease in size and numbei but not in density

Fig 1 Upper left The granular form of the tubercle bacillus stained by the Ziehl Neelsen technique, with its unstained wall outlined by nigrosine
Fig 2 Upper right Tubercle bacilli stained with carbol fuchsin containing sodium chloride   The dye is evenly distributed throughout the microorganism
Fig 3 Lower left The same cells as in Figure 2, washed with acid alcohol and then with water   The dye has been precipitated to certain points in the cell
Fig 4 Lower right The same cells as in Figure 3, washed with 95 per cent ethyl alcohol   The dye is again evenly distributed throughout the cell
All illustrations were taken at a magnification of 1,200 then enlarged three times

Furthei obser\ations support our contention that the formation of beads repre-sents an accumulation or precipitation of free dye within the tubercle bacillus

(1) The location of beads cannot be predicted before formation, nor can evi-dence of then previous location be found after dispersal with alcohol or phenol When reformed, beads may be found in their original location, but just as often they occupy new positions in the cell (Porter and Yegian, 1945)   These findings as well as the fact that the beads may be produced in cells from which the ether-

alcohol-soluble constituents have been removed, make it very unlikely that beads are formed about a pre-existing structure within a fixed organism

(2) The rate at which the precipitated dye is dissolved does not resemble the gradual withdrawal of dye from a stained structure   The addition of an electrolyte, such as sodium chloride, to a solution of carbol fuchsin results in the formation of a precipitate which, when separated by centrifugation, is readily soluble in alcohol

(3) The process of bead formation is almost instantaneous   Occasionally we have noted that beads were not formed so soon as water reached the preparation but that a slight tap on the bench supporting the microscope coincided with sudden bead formation   The effect of vibration on precipitation is well known

(4) The time necessary to cause beads to disappear is longer when stained films are kept for several weeks and then treated with alcohol   This suggest that the beads in older films contain less liquid

(5) The granular form of the tubercle bacillus may be stained to show beading but it will be noted that the dye accumulates and is dispersed only in the granular portions and not in the unstained interspaces   This finding is further support for the contention that in these granular cells the cytoplasmic membrane has separated and is about the individual granules which are retained in the cell by the cell wall

(6) The phenomenon of accumulation or precipitation of free dye may be observed within cells other than mycobacteria   Certain mushroom spores when stained resist decolorization   They may also show bead formation and subsequent dye dispersal when stained and treated by the method previously described

Our original hypothesis would be further supported if we could show that the free dye can be removed from the cell without altering its acid-fast property

The following method was employed to demonstrate the removal of free dye Two suspensions of tubercle bacilli, strain H37, were made   one in distilled water and one in which the bacilli were mixed with an approximately equal amount of "tween 80" (Dubos and Davis, 1946) previous to suspension in distilled water Both suspensions were boiled in a water bath for 20 minutes, and a loopful of each was placed on a slide   The films were dried in air and then stained by the usual Ziehl-Neelsen technique (Yegian and Budd, 1943)

All visible dye was removed from the films after immersion in either boiling neutral 50 per cent alcohol or boiling water   It was observed that the dye was removed from the films containing "tween" in 30 to 60 seconds as compared with 5 to 7 minutes for those which did not contain "tween"   The dye from both films was removed more rapidly by immersion in boiling alcohol than by immersion in boiling water

When the films from which all free dye had been removed were examined microscopically, the individual bacilli appeared faintly pink   To remove the last faint trace of dye absorbed by the cytoplasm required considerable longer immersion   The change in staining that occurred as the result of this immersion was so marked that one would not hesitate to say that the cell

acid-fast   When the cells are counterstained with brilliant green following the immersion procedure, they appear brilliantly stained, showing that the free dye which was removed was probably the source of the acid-fast coloring

When films of bacteria from which the free lipid had been extracted were prepared and stained as before and then immersed in boiling water or alcohol, the time required to remove the dye was not altered

We believe that the decolorization procedures described here have their basis in the alteration of the permeability of the cytoplasmic membrane by the boiling solutions, but that the change is not permanent is evident when the Ziehl-Neelsen procedure is repeated and the bacilli are found to be acid-fast   The action of "tween 80" is not understood, but it may, by altering the permeability of the cytoplasmic membrane, permit the more rapid removal of free dye

DISCUSSION AND SUMMARY

The foregoing observations lead us to propose an explanation for the acid-fast characteristic of mycobacteria that is both simple and comprehensive   Our hypothesis is supported by the fact that, during the staining procedure when the Ziehl-Neelsen technique is employed, fuchsin enters the cell through the cytoplasmic membrane and is not removed by the acid alcohol used in the procedure   Fuchsin exists within the cell in two forms   (1) One is a form which can be accumulated in beads and acts as free dye   It is to this form that the usual color of the stained acid-fast bacillus is attributed   (2) A small portion of the dye acts as though firmly bound by the cytoplasm and gives the organism only a very faint pink color

Lamanna (1946) has postulated that beading is the result of phenol and dye separating out as a liquid phase   We have been able to show that mycobacteria will exhibit acid-fastness and beading even after extraction of the free lipids   Since we have not noted any progressive decrease in intensity of acid-fast coloring during the stages of extraction, we do not believe that the acid-fast property lies in the greater solubility of phenol and dye in the cellular lipids than in the decolorizing agent

Anderson (1932) isolated mycolic acid and has brought evidence to show that mycolic acid exists as a lipopolysaccharide in the cell and that this complex is acid-fast   Long (1922) believed that bound lipid existed as a lipoprotein in the cell and that this complex determined cell permeability   Boissevain (1927) believed that acid caused hydrolysis of an acid-fast substance in the cell and thus loss of acid-fastness

In our experience and in that of Rich (1944) mycolic acid and the other extractable lipids are not strongly acid-fast, and when extremely thin films of mycolic acid are stained they appear only faintly acid-fast   Also, no mycolic acid or closely related substance has been extracted from certain acid-fast structures other than mycobacteria   The fact that lipids extracted from tubercle bacilli are only faintly acid-fast and that without drastic procedures, such as the

use of acid in the extraction, the bacilli remain acid-fast suggests that the relation of extractable lipids to acid-fastness has been overestimated   Although it is known that acid hydrolysis will split lipoprotein complexes in certain cells, it is also known that acid can affect a variety of other cellular components and struc tures, some of which may be important in the property of acid-fastness   Ex traction of a chemical from a cell may upset the function of a structure in the cell in some demonstrable way, but it does not necessarily follow that the extracted chemical or one of its complexes within the cell represents the major factor in the function of the structure   It may be true that certain lipo-complexes are con trolling factors in the permeability property of the cell, but in itself the permea bility function of the intact cytoplasmic membrane needs emphasis

We have shown that apparently only a small portion of the dye is bound to cy toplasm   The essential characteristics of the beading process, such as the visible shift of the dye within the cell, the variability of location of the beads in the same bacillus after dissolution and reformation, and the failure to demonstrate any structure about which the beads might be formed all substantiate the fact that the major portion of the dye behaves as would be expected of a free dye   The process of beading resembles precipitation very closely, and evidence has been offered for this idea

We have shown that the free dye, which gives the usual color to the stained bacillus, can be removed by immersion in boiling alcohol or water without alter ing permanently the acid-fast property

The fact that tubercle bacilli are rendered non-acid-fast by trauma, autolysis, or the use of acid is satisfied by our theory, the action has been so severe that the cytoplasmic membrane no longer controls permeability with relation to the dye used

The appearance of acid-fast mushroom spores stained by the Ziehl Neelsen procedure to show beading and the reaction of the resulting beads to phenol or alcohol give us some evidence that the existence of free dye that can be accumu lated and then redistributed within a structure is not peculiar to mycobacteria Certain acid-fast structures other than tubercle bacilli have been shown to con tain only small quantities of lipids   The theory we are proposing can account fo the acid-fastness of these structures as well as of the mycobacteria

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GROUP OF BACTERIA

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Instances of antagonism among the enteric group of bacteria have been frequently reported in the literature, but these are concerned primarily with mixed cultures in which many different factors, difficult to analyze, are involved In 1925 Gratia observed a strain of *Escherichia coli* which produced a diffusible and thermolabile antibiotic substance   He called this substance "principe V " This "principe V" seemed to be specific and at the time appeared to be active against only one other strain of *Escherichia coli* and *Shigella dysenteriae*   Later, Gratia and Fredericq (1947) found that antibiotic strains of *E coli* are not at all infrequent, but that the antibiotic spectrum of the different strains was characteristically different and distinct

The antibiotic substances produced, to which was assigned the term "colicin" (Gratia and Fredericq, 1947), differ not only in their antibiotic spectra but also in their physiochemical properties (Gratia and Fredericq, 1946)   It has been observed that some strains may produce several colicins with characteristically different antibiotic spectra   It was furthermore observed that practically every member of the *Enterobacteriaceae* studied, including the strains which themselves produced antibiotic colicins, was sensitive to one or more colicins produced by some other member of the *Enterobacteriaccae*

In the present study of antibiotic interrelationships among members of the enteric group of bacteria we employed the following technique (Fredericq, 1946) A small (about 1-mm) loop of a broth culture of a strain to be tested for production of antibiotics was stabbed on previously poured and dried peptone agar plates   After incubation for 48 hours at 37 C the culture was killed by exposure to chloroform vapors for a period of about 1 hour, and the chloroform was then allowed to evaporate   The entire surface of this agar plate was then inoculated with a strain being observed for sensitivity   A convenient procedure for this purpose consisted of covering the surface of the medium with a sterile filter paper and then inoculating this filter paper with a ml or two of a culture of the organism being studied for sensitivity   After contact for several minutes the filter paper was removed and the plate incubated for 24 hours at 37 C   If an antibiotic was produced, growth of a sensitive strain was inhibited in a circular zone in the vicinity of the stab but not restricted on the rest of the plate, as may be seen in figure 1   It will be observed that two strains were markedly antibiotic, one only very slightly, and the fourth showed no evidence of antibiosis against a strain of *Escherichia* being tested for sensitivity

The antagonistic action under consideration appears to be quite different

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from the autoinhibition of coliform bacteria reported by Powers and Levine
(1937) and Coblentz and Levine (1947) when employing the so called staling
technique, which consists of preparing agar by adding 1 3 per cent agar gel to an
equal volume of an old broth culture of the test organism and then observing
growth after reinoculation of this medium   Thus, from table 1 it will be
noted that *Escherichia* strain CA1 did not grow on a staled medium made with
that same culture, but it was not autoinhibitory when employing the antibiotic

FIG 1 ANTIBIOTIC ACTIVITY AMONG ENTERIC BACTERIA

technique described above   *Escherichia* strain C A2, *Shigella* C A11, and p
colon CA79 similarly show autoinhibition of growth by the staling technique,
but offer no evidence that a diffusible autoinhibitory substance is produced
when they are tested by the antibiotic technique   On the other hand ''
C A1, C A2, and C A44 show no evidence of inhibition against strain A9
the staling technique is employed, but marked inhibition when the antibi
technique is employed   Strain C A44 is particularly striking in that it
biotic against a number of other strains but not, however, against its own
two substances associated with growth inhibition—namely, that which

distinct until their nature is more definitely determined

ore, have to be considered as probably

TABLE 1

Growth inhibition on staled agar and in vicinity of colonies of antibiotic strains

STRAIN OBSERVED FOR GROWTH INHIBITION		Escher ichia CA1	Escher ichia CA2	Shigella* CA44	Para colon CA79	Escher ichia CA8	Escher ichia A9	Citro bacter CA33	Aero bacter A6
Antibiotic strains employed	Tech nique	Inhibition of growth							
Escherichia CA1	S	+	+	+	+	+	–	–	–
	A	–	–	–	~	–	+	–	–
Escherichia CA2	S	+	+	+	+	+	–	–	–
	A	–	–	–	–	–	+	–	–
*Shigella** CA44	S	+	+	+	+	+	–	–	–
	A	+	+	–	+	–	+	+	+
Paracolon CA79	S	+	+	+	+	+	–	–	–
	A	–	+	–	–	–	–	+	–

A, antibiotic technique (2 day culture, 37 C)
S, staled medium prepared from 10 day culture, 37 C
* Final allocation not definitely made

TABLE 2

Frequency of antibiosis among strains of enteric bacteria

STRAINS TESTED FOR GROWTH INHIBITION	GROUP	Escher ichia	Para colon	Citro bacter	Aero bacter	Pro teus	Salmo rella	Eber thella	Shigella	All Groups
	No	56	23	13	14	8	33	6	41	194
Antibiotic strains		Per cent of trials showing growth inhibition								
Group	No									
Escherichia	39	37	20	10	2	4	22	19	44	28(7,566)*
Paracolon	21	22	12	7	0	0	6	9	44	18(4,074)
Citrobacter	8	26	20	19	0	0	7	25	47	23(1,552)
Eberthella	5	15	8	17	0	0	0	17	11	10(970)
Shigella	15	17	7	1	0	0	12	14	36	13(2,895)
All groups	88	28 (4,928)*	15 (2,024)	9 (1,144)	1 (1,232)	2 (704)	12 (2,904)	14 (528)	40 (3,593)	22(17,057)

* Figures in ( ) indicate number of trials or observations (product of numbers of antibiotic and test strains)

Table 2 shows the frequency of the phenomenon of antibiosis among strains of enteric bacteria Eighty-eight strains were tested for antibiotic potency against 194 strains of the colon group (the latter including the test strains) Of 17,057 observations, evidence of growth inhibition was observed in 22 per

cent   Considering the sensitivity of 56 strains of *Escherichia* to antibiotics produced by other enteric bacteria, it will be noted that growth inhibition was observed most frequently (37 per cent of the trials) when *Escherichia* strains were tested against other *Escherichia* strains and least frequently (17 per cent) when they were tested against *Shigella* strains

The paracolon bacilli showed results similar to those observed for the *Escherichia*

The *Citrobacter* strains were most frequently inhibited by other strains of *Citrobacter* or by *Eberthella* strains, but it should be noted that the number of antibiotic strains, 8 and 5, respectively, are too few for final judgment

As a group, the *Shigella* strains seem particularly prone to be sensitive to antibiotic agents produced by enteric bacteria   Thus, growth inhibition was observed in 40 per cent of the trials with *Shigella* as compared to 28 per cent with *Escherichia* and 12 per cent with *Salmonella*

In the 14 strains of *Aerobacter* and 8 strains of *Proteus* studied, sensitivity was only occasionally observed, and then only to a few strains of *Escherichia*

The *Salmonella* strains were frequently sensitive to *Escherichia* (22 per cent of the trials showed growth inhibition), occasionally sensitive to *Citrobacter* and paracolon strains, but never sensitive to the antibiotic *Shigella* or *Eberthella* strains employed

In table 3 is shown the frequency of growth inhibition by and among enteric bacteria   Of 56 strains of *Escherichia* that were tested for sensitivity to various other enteric forms, a large proportion (25 to over 75 per cent) were sensitive to a large number of other *Escherichia* strains, whereas only a few were sensitive to *Shigella* or *Eberthella* strains

Among 14 *Aerobacter* strains tested for sensitivity against other members of the *Enterobacteriaceae*, only 3 (or less than 25 per cent) were inhibited by *Shigella* and that was due to a single *Shigella* strain, 25 to 49 per cent of the *Aerobacter* showed some evidence of sensitivity to *Escherichia* strains, but there again only a small proportion of *Escherichia* strains (8 per cent) were antibiotic

It will be seen from table 3 that the 33 strains of *Salmonella* tested were re sensitive to any of the strains of *Shigella* or *Eberthella* and only occasionally sensitive to some *Citrobacter* and paracolon bacilli, but *Escherichia* strains were frequently antibiotic against *Salmonella* strains, as indicated by 41 per cent of the 39 *Escherichia* cultures employed showing antibiotic action against 25 to 75 per cent of the *Salmonella* strains under observation

Considering the *Shigella* strains, it again will be noted that these were particularly susceptible to *Escherichia* strains (95 per cent of the 39 antibiotic *Escherichia* strains being effective against 25 per cent or more of the *Shigella* cultures)   They were also sensitive to strains of *Citrobacter* and to paracolon bacilli, but particularly to other *Shigella* strains (47 per cent of them being active against more than 50 per cent of the *Shigella* cultures tested for activity)

The insensitivity of *Proteus* strains is evident as only 2 (5 per cent) of the *Escherichia* cultures were antibiotic against them, but these *Escherichia* strains were effective against 6 of the 8 strains of *Proteus* observed

TABLE 3

Frequency of growth inhibition by and among enteric bacteria

ANTIBIOTIC ENTERIC STRAINS	Escherichia 39	Paracolon 21	Citrobacter 8	Eberthella 5	Shigella 15	ANTIBIOTIC ENTERIC STRAINS	Escherichia 39	Paracolon 21	Citrobacter 8	Eberthella 5	Shigella 15
Per cent of 56 *Escherichia* inhibited	Per cent of various strains antibiotic against *Escherichia*					Per cent of 13 *Citrobacter* inhibited	Per cent of various strains antibiotic against *Citrobacter*				
0						0	72	76	12		93
<25	31	81	50	100	100	<25	18	14	88	100	7
25–49+	54	14	50			25–49+		5			
50–74+	10	5				50–74+	2	5			
75+	5					75+	8				
Per cent of 23 paracolon inhibited	Per cent of various strains antibiotic against paracolon					Per cent of 14 *Aerobacter* inhibited	Per cent of various strains antibiotic against *Aerobacter*				
0	13				33	0	92	100	100	100	93
<25	69	90	75	100	67	<25					7
25–49+	10	10	25			25–49+	8				
50–74+	8					50–74+					
75+						75+					
Per cent of 33 *Salmonella* inhibited	Per cent of various strains antibiotic against *Salmonella*					Per cent of 41 *Shigella* inhibited	Per cent of various strains antibiotic against *Shigella*				
0	26	67	75	100	100	0	2				7
<25	33	24				<25	3	14	12	100	40
25–49+	28	9	25			25–49+	77	81	50		6
50–74+	13					50–74+	8	5	38		47
75+						75+	10				
Per cent of 8 *Proteus* inhibited	Per cent of various strains antibiotic against *Proteus*					Per cent of 6 *Eberthella* inhibited	Per cent of various strains antibiotic against *Eberthella*				
0	95	100	100	100	100	0	36	67			80
<25						<25	54	23	75	100	20
25–49+						25–49+		5	25		
50–74+						50–74+		5			
75+	5					75+	10				

per cent of the 56 *Escherichia*, 70 per cent of 23 paracolon bacilli, 85 per cent of 13 *Citrobacter* strains, 100 per cent of 6 *Eberthella* strains, and 80 per cent of 41 *Shigella* strains, compared with only 27 per cent of the 33 *Salmonella* strains It was entirely inactive against the 7 *Proteus* and the 14 *Aerobacter* strains that were observed

The growth inhibition zones varied markedly for different strains, indicating a large variation in the degree of resistance or susceptibility of the various strains tested to the antibiotics produced by this *Escherichia* strain (37)   Perhaps a particularly striking observation is that among the 33 strains of *Salmonella* tested for sensitivity to this strain of *Escherichia*, 24 (73 per cent) were insensitive, but for the 9 sensitive strains the diameter of the inhibition zone was over 40 mm in eight instances (in three of which it was over 50 mm in diameter) The 9 highly sensitive strains of the *Salmonella* included an unidentified strain,

TABLE 4

Growth inhibition zone against various enteric bacteria

DIAMETER INHIBITION ZONE		NONE	<10 MM	10-19 MM	20-29 MM	30-39 MM	40-49 MM	50-59 MM	PER CENT STRAINS INHIBITED†
Test strains		Number and per cent* of test strains inhibited by *Escherichia* strain 37							
Group	No								
Escherichia	56	7 (13)	1 (2)	10 (18)	7 (12)	26 (46)	5 (9)		85
Paracolon	23	6 (26)	1 (4)	4 (17)	4 (17)	2 (9)	6 (26)		70
Citrobacter	13	2 (15)		2 (15)	3 (23)	5 (39)	1 (8)		85
Aerobacter	14	14 (100)							
Proteus	7	7 (100)							
Salmonella	33	24 (73)				1 (3)	5 (15)	3 (9)	27
Eberthella	6			5 (83)		1 (17)			100
Shigella	41	8 (19)	2 (5)	13 (32)	12 (29)	5 (12)	1 (2)		80
		Number and per cent* of test strains inhibited by *Shigella sonnei* strain P 9							
Escherichia	56	42 (75)	2 (4)	5 (9)	4 (7)	3 (5)			21
Paracolon	23	19 (83)	1 (4)	2 (9)	1 (4)				13
Citrobacter	13	11 (84)		1 (8)	1 (8)				16
Aerobacter	14	13 (93)		1 (7)					7
Proteus	7	7 (100)							
Salmonella	33	33 (100)							
Eberthella	6	5 (83)	1 (17)						
Shigella	41	12 (29)		5 (12)	22 (54)	2 (5)			71

* Per cent to nearest whole number
† Showing inhibition zones of at least 10 mm

the only *S tennessee* and *S newport*, each of the two *S enteritidis*, and the four *S schottmuelleri* of our collection   It would be interesting, and perhaps significant, to determine whether other strains of these *Salmonella* types are similarly sensitive to *Escherichia* strain no 37

The inhibition zones produced by the *Shigella sonnei* strain (P9) were very much smaller than those frequently observed with the *Escherichia* strain referred to above, and it will be noted that it was effective particularly against other strains of *Shigella*, 29 (or 71 per cent) of the 41 *Shigella* strains tested for sensitivity showed inhibition zones

As illustrated by these two examples, the antibiotic spectra of various active

~~~~~ ~s may differ widely even among members of the same group   However, when a strain of a well-defined type of *Salmonella* or *Shigella*, such as *S schottmuelleri* or *Shigella sonnei*, was found to be sensitive to one or more of the active cultures, all other strains of the same type available at the time of this study were likewise susceptible to the antibiotics produced by those particular active cultures   This indicates that there may be some correlation between biochemical properties, or antigenic structure, and antibiotic sensitivity

SUMMARY

The results obtained with 88 antibiotic enteric strains against 194 members of the *Enterobacteriaceae* (including the antibiotic strains), employing a simple technique for rapidly ascertaining sensitivity or antibiotic activity, are described

Members of the genus *Shigella* were more frequently sensitive to antibiotics produced by various enteric strains than were other genera of the *Enterobacteriaceae*

The frequency of sensitivity to antibiotics produced by various members of the *Enterobacteriaceae* decreased in the following order   *Shigella, Escherichia,* paracolon bacilli, *Citrobacter, Salmonella, Proteus,* and *Aerobacter*   Thus, 40 per cent of 3,593 trials employing strains of *Shigella*, 28 per cent of 4,928 trials with *Escherichia*, 15 per cent of 2,024 trials with paracolon bacilli, 12 per cent of 2,904 trials with *Salmonella*, 2 per cent of 704 trials with *Proteus*, and 1 per cent of 1,232 trials with members of the genus *Aerobacter* showed evidence of sensitivity to antibiotics produced by members of the *Enterobacteriaceae*

Shigella strains were frequently active against *Escherichia*, particularly active against other *Shigella* strains, only occasionally active against *Citrobacter*, and entirely inactive against members of the genera *Aerobacter, Proteus,* or *Salmonella*

Escherichia strains, on the other hand, are not only active against many other strains of *Escherichia, Citrobacter*, paracolon bacilli, and *Shigella*, but also against many *Salmonella,* though only occasionally against strains of *Aerobacter* or *Proteus*

When a well-defined type of *Salmonella* or *Shigella*, such as *Salmonella schottmuelleri* or *Shigella sonnei*, was found to be sensitive to one or more active cultures, all available strains of the same type were similarly sensitive to those active cultures, indicating that there may be some correlation between biochemical properties, or antigenic structure, and antibiotic sensitivity

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